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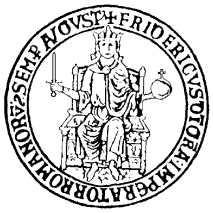

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The relevance of soil sampling, bioavailability assessment and amendment application in the remediation of soils polluted by potentially toxic metals (PTMs)

Ph. D. dissertation by

Claudia Rocco

Tutor: Prof. Paola Adamo

Co-Tutor: Prof. Massimo Fagnano

Co-Tutor: Dr. Nunzio Fiorentino

Coordinator: Prof. Domenico Carputo

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SUMMARY

Soil pollution by potentially toxic metal(loid)s (PTMs) is a globally widespread problem because of most metals don't undergo microbial or chemical degradation, and their total concentration in soil persists for a long time after their introduction. For these reasons is necessary to find soil remediation technologies, also to minimize the potentially accumulation of metals in the food chain. During the last decades, the application of the "green technologies" found the interest of the scientific community because more cost-effective and easily applicable. In this thesis, the importance of an appropriate sampling scheme to assess the PTMs pollution in agricultural soils subjected to waste disposal and dumping, the relevance of bioavailability assessment for phytoremediation and the potential PTMs (im)mobilization by amendments application were studied.

The choice of a most appropriate sampling scheme is a crucial step to establish soil pollution and, consequently, risk management. The sampling scheme should be able to detect the pollution level, determine its geographical extension and, possibly, the background level of pollution in the area. Usually, the high cost of the laboratories analysis and the difficulty of managing extensive soil sampling, lead to choose wide grids of sampling (from 25x25 m to 5x5 km) and to bulk discrete soil samples to obtain composite samples. However, when strong concentration gradients exist in the field compositing the discrete soil samples may significantly underestimate the risk posed by the pollutants. For this reason, the degree and spatial variability of soil pollution by PTMs, in three agricultural sites, subjected to waste disposal, were assessed applying a soil sampling scheme based on a two-level grid resolution. On the first level, a regular low-resolution 10x10 m grid was defined. On the second level, each grid was subdivided into nine high-resolution 3x3 m subplots. Discrete soil samples were taken from each 3x3 m plot. Composite soil samples were made bulking aliquots from the discrete soil samples. Soil samples were collected from both 'topsoil' (0-30cm) and 'subsoil' (30-60 cm) layers to evaluate vertical gradients. Comparing the statistical analysis and various pollution indices data from composite and discrete soil samples, only Giugliano (GI) site resulted to be polluted . Indeed, from the analysis of composite data, GI site appeared to be slightly polluted by Cu and Zn (mean content of 131 and 95 mg kg^{-1}, respectively). When the same analysis and indices were applied to discrete soil data, the slightly polluted site became highly polluted by Cu (mean and max of 276 and 1707 mg kg^{-1}) and Zn (174 and 972 mg kg^{-1}), and slightly polluted by Sb and As (max of 15 and 30 mg kg^{-1}). Furthermore, a large inhomogeneity in soil pollutant spatial distribution emerged. Most part of existing legislations on soil remediation, define an area as potentially polluted on the basis of the total or pseudototal content of PTMs. However, to establish the risk associated to soil PTMs pollution, it is crucial to determine the mobility and bioavailability

of pollutants, the latter defined as the fraction of the total metal(loid)s available to the receptor organism. Based on the previous work results, the mobility and bioavailabilityof Cu and Zn in polluted discrete soil samples was investigated applying single and sequential extraction procedures. Main aim of this work was, to assess the feasibility and the effectiveness of a phytoremediation action of the soil based on Eucalyptus planting assisted by compost, *Trichoderma sp*. and microorganisms. 1M NH_4NO_3 and 0.05M EDTA extractions were applied, before and during the phytoremediation treatment, to assess the readily and potentially bioavailable amounts of Cu and Zn. The amount of both metals extracted by 1M NH_4NO_3 was from 0.1 to 7.9 % of respective total contents while the amount extracted by 0.05 M EDTA ranged from 13 to 64 % of total content. After one year from planting, a significant reduction of Cu and Zn bioavailable amounts was observed, without any difference between the several applied treatments. Plants uptake was not significantly correlated with both chemical extractions and a general underestimation of 1M NH_4NO_3 bioavailable Zn in comparison with plant uptake was observed. Both single and sequential extractions suggested an higher mobility of Zn respect to Cu in soil. A reduction of the plant uptake efficiency, as assessed by the accumulation factor in correspondence of highly polluted plots was observed. Overall, in the study sites currently under assisted phytoremediation, the chosen treatments appear to be effective in reducing at least the mobile and plant available PTMs content of the soil.

When it is not possible to remove the metal(loid)s from polluted soils, other options, such as immobilization treatments should be considered as an integral part of risk management. Immobilization technique consists in an increasing of PTMs retention on soil. In agriculture, several amendments are frequently used to improve the soil properties. Many of them may modify significantly the mobility of PTMs in the soil environment. Part of the thesis work was, therefore, focused on the influence of organic and inorganic amendments on the bioavailability of As and Cd in two Australian soils with different physico-chemical properties. Soils were spiked with As and Cd and, after four weeks, incubated with poultry manure (PM), poultry manure biochar (PMBC) and coal fly-ash (CFA). After other four weeks, the incubated samples were analysed for pH and bioavailability of As and Cd, assessed by1M NH_4NO_3 extraction and pore-water analysis. These data were compared with plant uptake, using maize (*Zea mays* L.) as test crop. Cadmium was almost completely immobilized in all treated soils, mostly due to the influence that the amendments had on soil pH. Indeed, the higher pH of soil A (8.5±0.03), automatically reduced the bioavailability of Cd, independently of the applied treatment. Opposite behaviour was observed for As, whose bioavailability generally increased after amendments addition. Indeed, in soil B with pH 5.1±0.03, the As bioavailability was lower than in soil A and the application of amendments always increased

the bioavailable fraction. These trends were confirmed by maize uptake. In conclusion, the application of amendments to reduce the mobility and bioavailability of metal(loid)s in polluted soil resulted efficient for Cd and inefficient for As. This different behaviour was mainly related to their different nature (cation and anion, respectively) and intrinsic properties. Hence, the amendments, modifying soil characteristics, can influence the metal(loid)s mobility and bioavailability but their effect is related to the metal properties.

A similar experiment was carried out with soil and sludge from the ex-ILVA brownfield area located north of Naples city (south Italy). A phytoremediation study was carried out for two consecutive years, involving the use of humic acids to enhance the adaptability and grown of *A. Donax* on polluted soil and post-washing sludge with attention to the mobility and bioavailability of PTMs. The humic acid application significantly increased the biomass production, in both soil and sludge. In particular, although *A. donax* grown on the most polluted substrate, i.e. sludge, it was interesting to observe an increasing of plant biomass as a consequence of humic acid addition, maybe due to an improvement of rhizosphere N cycling activity consequent to more intense root activity and exudation. The addition of humic acid, although useful for the plant growth, had no influence on Pb and Zn mobility and bioavailability, in both soil and sludge, probably as a consequence of the high pH value of both substrates. Despite the low bioavailability of the metals in soil and sludge, *A. donax* uptaken more Pb and Zn than the amounts "predicted" by 1M ammonium nitrate extraction, confirming the high ability of *A. donax* to phytoextract metals in all conditions and, hence, encouraging its use for phytoremediation purposes.

INTRODUCTION

The term "heavy metals" is referred to elements with density above 5 g/cm^3, with cationic behaviour and great tendency to form stable complex with organic matter. Generally, low heavy metal concentrations are essential for plants growth and human health, but high dosage can cause severe toxicity, like cancer, or mutagenic and teratogenic problems and endocrine disruptors while others cause neurological and behavioral changes especially in children, and for this reason heavy metals are also known/called as potentially toxic metals (PTMs).

Soil pollution by potentially toxic metal(loid)s (PTMs) is a globally widespread problem and many international researchers try to develop monitoring methods and sustainable technologies of polluted soils remediation. Soil is a non-renewable resource and its formation and development is closely related to soil genesis factors (natural and anthropogenic) (Washa *et al*., 2014). Furthermore, soils acts as a long term sink for PTMs released into the environment by anthropogenic activities and, unlike organic contaminants, most metals don't undergo under microbial or chemical degradation, and their total concentration in soil persist for a long time (from hundreds to thousands of years depending on the element and the properties of the soil) after their introduction (Bolan *et al*., 2014; Megharaj *et al*., 2011; Nicholson *et al*., 1999). Thus, remediation of PTMs pollution deserves due attention. However, changes in their chemical forms (speciation) and bioavailability are possible and the presence of toxic metals in soil can severely inhibit the biodegradation of organic contaminants (Wuana and Okieimen, 2011). For these reasons is necessary to find soil remediation technologies, also to minimize the potentially accumulation of metals in the food chain (de Abreu *et al*., 2012).

The objectives of remediation processes are usually based on threshold levels (or trigger concentrations) of soil contaminants (García Frutos *et al*., 2012; Van Nevel *et al.*, 2007), above these values, remedial measures and treatments are necessary. Indeed, the success of remediation occurs when the concentration of metals in polluted soil is bring back to the threshold values of current environmental regulations (sometimes, agricultural soils are not included). The guidelines for threshold levels, "trigger concentrations," of metals, differed from place to place to meet the variable ecological conditions of each region or country.

Knox *et al*. (1999) made a distinction between soil contamination and soil pollution. The first is defined as soil whose chemical state deviates from the normal composition but does not have a detrimental effect on organisms. Pollution occurs when an element or a substance is present in greater than natural (background) concentrations as a result of human activity and has a net detrimental effect on the environment and its component.

With term of background is intended the geogenic natural of metals in soil. The definition of background values, in contrast to baseline values (the current background levels), is very important in determining the extent of polluted areas in countries where environmental legislation has not yet established intervention limits for agricultural soils (Albanese *et al*., 2007).

Relevance of soils sampling

Risk assessment is an effective scientific tool which enables to manage sites polluted in a cost-effective manner while preserving public and ecosystem health (Wuana and Okieimen, 2011).

The choice of a most appropriate sampling scheme, is one of the key (or an important aspect), for better understand the "real" contamination degree (to locate areas that required remediation) and apply the best remediation technique.

The sampling scheme should be able to detect the pollution/contamination level, determine its geographical extension and, possibly, the background level of pollution/contamination of the site. Generally, the high cost of the laboratories analysis and the difficulty of managing wide soil sampling, lead to choose a low-resolution scale of sampling. Several authors for characterize an area, e.g., used grids from 5x5 km (Bove *et al*., 2011) to 25x25 m (Correll, 2001). Using this sampling scales, is reasonable to doubts on the possibility of undergo or, worst, don't see the correct pollution degree.

To characterize an area, could be useful the application of pollution indices. Index like pollution index (PI), pollution load index (PLI), geoaccumulation index (I_{geo}), enrichment factors (EF), contamination degree (C_{deg}) and so on, were applied to several authors (Li *et al.*, 2014; Kalender *et al.*, 2013; Loska *et al.*, 2004) to assess the soil environmental quality. Appropriate soil sampling and pollution assessment are also crucial for assessing soil volumes requiring remediation.

PTMs bioavailability assessment and fractioning

Naidu *et al.* (2008) defined bioavailability of metal(loid)s in the soil environment as the fraction of the total metal(loid) in the interstitial pore water (i.e., soil solution) and soil particles that is available to the receptor organism.

Most of the existing legislation on soil remediation agree on the limits based on total concentration only (Keller and Hammer, 2004), but risks management based only on the total or pseudototal content of PTMs in soil, might minimise the risk (Adamo and Zampella, 2008). The environmental behaviour of PTMs in soils and potential risks to human health depend on the form in which they occur and the concentration of soluble fraction. The manner in which metals is bound to the components of the soil complex, influences the mobility and the bioavailability of the metal to

organisms. The availability depends on the nature of chemicals association between the metal and organic and/or inorganic soils constituents (Janoš *et al.*, 2010). Hence, important is define the pollutants present in a form able to be absorbed by the body or is mobile and able to be taken up into plants (Markus and McBratney, 2001).

Soil are heterogeneous and complicated mixture. PTMs react with soil constituent in various ways. Chemical and biochemical processes (precipitation, adsorption, complexation, oxidation, and so on) are important in control of metals mobility and bioavailability in soil. All these processes are affected by soil pH and biological processes. The mobility of metals depends not only on the total content of these in soil but also on soil characteristics (e. g., pH, organic matter, texture, cation exchange capacity), metal properties and environmental factors (Bakircioglu *et al*., 2011; Irene *et al*., 1997).

Knowledge of the basic chemistry, environmental and associated health effects of these PTMs is necessary in understanding their speciation, bioavailability and remediation options. The fate and the transport of PTM in soil depends significantly on the chemical form and speciation of the metal. Depending on the form of metal bound with the soil, PTMs are accumulate in soils in various forms: water-soluble, exchangeable, oxide-bound, carbonate-bound, organic matter-bound and residual that is bound to resistant minerals (silicates) and non-extractable. The metals present in these forms have different mobility and bioavailability. Water-soluble and exchangeable fractions are readily released to the environment, but the residual fractions are immobile under natural conditions (Bakircioglu *et al*., 2011; Wuana *et al*., 2011).

The fraction of metal total content in soil that can be absorbed by plants via root-uptake defines the plant-bioavailable fraction (Adamo and Zampella, 2008; Kabata-Pedias and Pedias, 2001).

Single (one extranet solution and one soil sample) and sequential chemical extractions (several extraction solution are used sequentially to the same sample) procedures are commonly used in fractionation study and to predict the bioavailability of metal(loid)s in soil simulating the plant-bioavailable PTMs content. Chemical extractions are mainly based on extractions by different solution: dilute mineral or organic acid (acetic acid, $HCl+HNO_3$), chelating agents (Na_2EDTA, EDTA, DTPA), buffered salts (CH_3COONH_4) or neutral salts ($CaCl_2$, NH_4Cl, NH_4NO_3). This speciation methodologies use a single extractants to dissolve soil phases, chemical forms or binding types whose element content correlates with the availability of the element to plants (Adamo and Zampella, 2008).

According to Rauret (1998), single extractants may be divided into three main groups: exchangeable (unbuffered salt solution), organically complexed metal fraction (chelating agents), and strong acid-extractable fraction (acid extraction). The first type of extractants includes weakly

adsorbed metals retained on the solid surface by relatively weak electrostatic interaction, metals that can be released by ion-exchange processes. This fraction can be considered as bioavailable. The unbuffered salt solutions ($CaCl_2$, $NaNO_3$, NH_4Cl, NH_4NO_3, $AlCl_3$, $BaCl_2$) are rapid and simple extractants to extract bioavailable metals. But they do not represent plant available metals (Gupta and Aten, 1993). The chelating agents, such as EDTA and DTPA can displace metals from insoluble organic or organometallic complexes in addition to those sorbed on inorganic soil components. Chelating agents dissolve not only exchangeable element fraction but also the element fraction fixed on the soil hydroxides. The organic fraction released in the oxidizable fraction is not considered very mobile or available since it is associated with stable high molecular weight humic substances that release small amounts of metals in a slow manner. Acid extraction may involve strong mineral acids (HCl, HNO_3) or weak organic acid (acetic acid). Digestions in strong acids do not dissolve the silicate matrix. Such reagents do not mobilize trace elements from silicate parent materials but dissolve metal pollutants which largely enter the soil environment in non-silicate bound forms (Bakircioglu *et al*., 2011).

In details, in this work, 1M NH_4NO_3 and 0.05 M EDTA solution were used to PTMs bioavailability assessment. First solution, called also soft extractant, is a weak extractant that extracts mainly the water soluble and part of the exchangeable fraction of metals (Wu et al., 2004). The key rule is played by dissociation of NH_4^+. Usually, metals have a good affinity to form metal ammine complex. In Germany, this method has been published as an official standard (DIN 19730, 1997). Whilst, EDTA (strong chelating agent) can form stable chelates with many metal ions, can remove organically bound metals along with metals occluded in oxides and secondary clay mineral part (and is resistant also to microbial degradation). The EDTA is a non-specific extractant and can remove both labile and non-labile fractions. When a statistically significant linear correlation exits between metal ion content in soil plant-available extracts and in plants, then it is possible to evaluate the soil-plant transfert factor for given metal (Adamo and Zampella, 2008; Irene *et al*., 1997).

Several authors, starting from Tessier *et al.* (1979), applied sequential extraction procedures to study polluted soils. Sequential extraction procedures involve treatment of a soil sample with a series of reagents, with increased extractants strength, in order to portioning PTMs content. The extractants (three to seven steps, or nine in more sophisticated analysis-Krishnamurti and Naidu (2000)) are generally applied as follow: unbuffered salts (soluble), weak acids (exchangeable), reducing agents (precipitate), oxidising agents (organic) and strong acids (occluded). The principal advantage of sequential extraction over the use of single extractants is that the phase specificity

improve. Specific pools measured by chemical fraction schemes have been correlate with plant uptake of PTMs (Bolan *et al.*, 2014).

The use of both single and sequential extraction procedures may provide useful information for both short and long-term biological uptake of PTMs.

On these criteria were developed several strategies, like phytoremediation techniques, or the use of amendments, to reduce risk associated with the presence of PTMs in soils and to minimize potential impact on plants, animals, water quality and human health.

Plants and soil amendments for remediation

Methods for remediating PTMs-polluted soils have been widely investigated and discussed. Several techniques, are applied to cleanup soils polluted with PTMs as described by Kabata-Pedias (2011).

One of the problem in soil remediation actions is the cost. Phytoremediation techniques are likely to be less costly than those based on conventional technologies. At present, a requirement for phytoextraction is to increase the yield of plants that hyperaccumulate metals from soils and to develop adequate technologies for the utilization of plant materials. Agricultural practices also have been applied to soil remediation. Most commonly, the uptake of metals by plants is diminished by keeping a neutral soil pH and by amendments with materials having a high capacity to bind metals in slightly mobile fractions (Kabata-Pedias, 2011).

In this thesis, phytoremediation and (im)mobilization assisted by amendments application were studied. Phytoremediation can be defined as an in situ remediation strategy that uses vegetation and associated microbiota, soil amendments, and agronomic techniques to remove, contain, or render environmental pollutants harmless. The idea of using metal-accumulating plants to remove PTMs and other compounds was first introduced in 1983. Phytoremediation is a relatively new technology and is still mostly in its testing stages. However, it has been tested successfully in many places around the world for many different contaminants. This technique, is energy efficient, aesthetically pleasing method of remediating sites with low to moderate levels of pollution, and it can be used in conjunction with other more traditional remedial methods as a finishing step to the remedial process. Phytoextraction is a part of phytoremediation techniques, and consist in plant roots uptake PTMs from the soil and translocate them to their tissues. A plant used for phytoremediation needs to be metal(loid)s tolerant, grow rapidly with a high biomass yield per hectare, have high metal-accumulating ability in the foliar parts, have a profuse root system, and a high bioaccumulation factor (Wuana and Okieimen, 2011).

When is not possible remove the metal(loid)s from polluted sites by phytoremediation, other options, such as immobilization should be considered as an integral part of risk management (Bolan

et al., 2014; Park *et al*., 2011). Immobilization technique consist in an increasing of PTMs retention on soil or on the decrease the PTMs mass transfer rate from the soil matrix (Irene *et al.*, 1997). In agriculture, various amendments, both inorganic and organic, are frequently used to improve the soil properties, such as microbial and enzyme activities, or properties of soil organic matter (Lima *et al.*, 2009). Many of them may modify significantly the mobility of PTMs (and also nutrients) in the soil environment. Soil organic matter that plays a key role in governing the metal mobility consists mainly of humic substances-humic and fulvic acids.

Several authors (Bolan *et al.*, 2014; Kumpiene *et al.*, 2008; Naidu *et al*., 2006; Adriano *et al*., 2004; Basta *et al*., 2001; Knox *et al*., 2000) have described effects of natural attenuation, using agricultural chemicals (e.g., lime, phosphate compounds, secondary minerals, Fe-oxides, organic compounds) in reducing mobility and thus phytoavailability of metals. The spread of contaminants in soil can be hindered by the soil stabilization technique, which is based on an application of suitable immobilizing agents. Adsorption of contaminants on mineral surfaces, formation of stable complexes with organic ligands, surface precipitation and ion-exchange were identified as the main mechanisms responsible for the reduction of the metal mobility, leachability and bioavailability. The soil amendments for the stabilization of As, Cr, Cu, Pb and Zn in soils were reviewed recently by Kumpiene *et al.* (2008). Park *et al.* (2011) had shown that the addition of organic amendments to soils increases the immobilization of metals through adsorption reactions. The organic amendments induced retention of PTMs is attributed to an increase in surface charge and the presence of metal(loid)s binding compounds. Organic amendments like compost, farmyard manure, may reduce the bioavailability of PTMs in soils due to its high content of humified organic matter (OM) and high concentration of P and Fe. The effect of manure on PTMs availability, even if temporary, is due to the introduction of organic matter to the soil and thus permitting the re-establishment of vegetation on contaminated sites (Castaldi *et al.*, 2005). In contrast to the classical remediation techniques, immobilizing techniques alleviate contaminant exposure without destruction of soil structure and biological activity. Among immobilizing agents, compost is becoming increasingly widespread for its low cost and beneficial effect on soil fertility, aiding the re-establishment of vegetation and contaminates sites (Castaldi *et al.*, 2005; Chen *et al.*, 2010).

Inorganic amendment like lime, zeolite, coal are very effective in decreasing the metal bioavailability due to the introduction of additional binding sites for PTMs and pH effects. For example, coal fly ash (available in great quantities from coal-fired power plants) is a useful ameliorant that may improve the physical, chemical and biological properties of soils (Jala and Goyal, 2006). In soil remediation, it can be used alone, or, more often in a mixture with other amendments, such as quicklime (Dermatas and Moon, 2006), peat (Kumpiene *et al.*, 2007) or

sewage sludge (Su and Wong, 2003). Natural (less often synthetic) zeolites were also used successfully in the remediation strategies to immobilize PTMs in soils (Janoš *et al.*, 2010).

In contrast to the classical remediation techniques, immobilizing techniques alleviate contaminant exposure without destruction of soil structure and biological activity (Castaldi *et al.*, 2005; Chen *et al.*, 2010). But, immobilization means that the metals are present in the soils. The soil doesn't return to its original state and the long term stability and effects on plants and animals are unknown. Immobilization is thus not a permanent solution (Irene *et al.*, 1997).

Aims of the work

Aims of this work were:

a) to investigate the influence of different soil sampling approaches on PTMs pollution assessment, highlighting the difficulties to appropriately assess the level of pollution of agricultural soils interested by waste disposal when only composite soil sampling is applied;

b) to assess the PTMs mobility and bioavailability, using single and sequential chemical extractantion procedures, in order to predict the feasibility of phytoremediation treatments in agricultural polluted soils and to investigate, after one year, the phytoremediation effectiveness;

c) to evaluate the influence of several organic and inorganic amendments on PTMs mobility/bioavailability and plants growth in agricultural soils with different pH and in industrial soil and sludge.

CHAPTER I

Composite *vs.* discrete soil sampling in assessing soil pollution of agricultural sites interested by waste disposal

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Claudia Rocco*[1,2], Ida Duro[1], Salvatore Di Rosa[3], Massimo Fagnano[1,2], Nunzio Fiorentino[1,2], Angela Vetromile[3], Paola Adamo[1,2]

[1]*Department of Agricultural Sciences, University of Naples Federico II, Portici (Naples), Italy*

[2]*CIRAM–Interdepartmental Centre for Environmental Research, University of Naples Federico II; via Mezzocannone, 16, 80132 Naples, Italy;* paola.adamo@unina.it

[3]*ARPAC – Campania Region Environmental Protection Agency, Naples, Italy*

*Corresponding Author: claudia.rocco@unina.it

Abstract

The choice of an appropriate sampling scheme is a crucial step in the process of soil pollution assessment and risk management. In agricultural systems, where soil is mixed by ploughing, the bulking of discrete samples to obtain composite samples improves soil sampling precision, unless strong concentration gradients exist. In this case, the compositing may significantly underestimate the risk posed by the contaminants. In this paper, the degree and spatial variability of soil pollution by potentially toxic metals in three agricultural sites, subjected to unauthorized waste disposal, were assessed applying a soil sampling scheme based on a two-level grid resolution. On the first level, a regular low-resolution 10x10 m grid was defined. On the second level, each grid was subdivided into nine high-resolution 3x3 m subplots. Discrete soil samples were taken from each 3x3 m plot. Composite soil samples were made bulking aliquots from the discrete soil samples. Soil samples were collected from 'topsoil' (0–30cm) and 'subsoil' (30–60cm) layers to evaluate vertical gradients. When statistical analysis and various pollution indices were applied to composite data, only one site appeared to be slightly polluted by Cu and Zn, with mean content of 131 and 95 mg kg^{-1} and peaks of 275 and 174 mg kg^{-1}. When the same analysis and indices were applied to discrete soil data a much worse scenario emerged. The slightly polluted site became highly polluted by Cu (mean and max of 276 and 1707 mg kg^{-1}) and Zn (174 and 972 mg kg^{-1}), and slightly polluted by Sb and As (max of 15 and 30 mg kg^{-1}). Plots classified as unpolluted on the base of composite data revealed metals above legal limits. Pollution always interested both topsoil and subsoil, which in few cases showed higher values than in topsoil. The adopted two-level soil sampling scheme succeeded to show dishomogeneity in soil pollutant spatial distribution, with pollution foci emerging only when sampling was done at a very short spatial scale.

Keywords: Heavy metals, soil pollution, spatial distribution, soil sampling, pollution indices

1. Introduction

Contamination of soil with potentially toxic metals (PTMs) in agricultural lands represents a serious risk to human and animal health due to the potential accumulation of pollutants in the food chain (Wuana and Okieimen, 2011). Moreover, in all parts of the world, agriculture is a primary sector of economy playing a key role in food security and rural environment sustainability, and any abandonment or change of land use would result in increased environmental pressures and deterioration of valuable farm habitats with serious economic and social consequences (Washa *et al.*, 2014). Hence, the adequate choice of sampling scheme, assessment of the level and geographical extend of soil contamination and, as consequence, the adoption of the most appropriate remediation strategy, is of vital importance in crop lands (de Abreu *et al.,* 2012, Loska *et al.,* 2003).

The determination of PTMs total or 'pseudototal' content in soil is considered a valuable preliminary aid in establishing the risks for biota and human health, assuming that pollutants transferring to water resources or biota are correlated with contamination level (Adamo and Zampella, 2008). In contrast, relevant paradigms in environmental monitoring, risk assessment and remediation feasibility are the natural levels of PTMs in soil, the spatial variability of soil pollution, the mobility and bioavailability of pollutants to microorganisms, plants, animals and humans. In an agricultural system, where the soil is homogenised by ploughing, the bulking of discrete samples, taken from several points of field plots, to form composite samples is a widely applied technique of soil sampling. The PTMs total content measured on the composite soil samples can be considered representative of the sampled field plots. Nevertheless, if in the soil there are strong horizontally or vertically gradients in terms of pollutant concentration, the compositing scheme may lose important information with a significant underestimation of the risk posed by the contaminant. In this case, different strategies of soil sampling have to be used in order to appreciate the spatial variability of soil pollution and to locate the areas that effectively require remediation (Correll, 2001).

Excavation, addition of extraneous materials and mixing of the soil matrix are frequent in the urban and peri-urban areas as a consequence of the intensive use of the territory and the rapid land-use changes (Ajmone Marsan and Zanini, 2013). In agricultural areas, the importance of soil as a medium for waste disposal is increasing with increase in industrialization and population (Lal and Pimentel, 2007). Intense anthropogenic activities, in agricultural as in city environments, add to the natural spatial variability thus intensifying soil heterogeneity (Behera and Shukla, 2014; Li *et al.*, 2011). The spatial variability of PTM pollution in agricultural soils is important for designing site specific agricultural and environmental soil and crop management practices. The scales of spatial

variation may differ between different soil properties, because the processes that cause variability may occur at different scales, e.g. from the single plant scale to larger topographical scales (Zhang *et al*., 2014). Understanding the patterns and processes of soil spatial variability is key for an efficient soil resource management. Disregarding spatial variability may cause undesirable results.

Main aim of this study was to highlight the difficulties to appropriately assess the level of PTMs pollution in agricultural soils interested by illegal waste disposal when only composite soil sampling is applied. For this reason, in three agricultural sites, subjected to unauthorized waste disposal, we applied a soil sampling scheme based on a two-level grid resolution. On the first level, a regular low-resolution 10x10 m grid was defined. On the second level, each grid was subdivided into nine high-resolution 3x3 m subplots. Discrete soil samples were taken from each 3x3 m plot. Composite soil samples were made bulking aliquots from the discrete soil samples. Soil samples were collected from both 'topsoil' (0–30cm) and 'subsoil'(30–60 cm) layers to evaluate vertical gradients. Data concerning the total content of PTMs in composite and discrete soil samples were compared using classical statistical methods to provide evidences of the potential loss of information when only composite samples are used. The need for soil-specific sampling guidelines for contamination assessment to reduce the risk arising when a high level of contaminant is missed is discussed.

2. Materials and methods

2.1 Study area

The Litorale Domitio Agro Aversano is an area of Campania Region (southern Italy), with a total surface of about 1564 km^2 (Bove *et al*., 2011,Capra *et al*., 2014), encompassing the plains of the Garigliano and Volturno Rivers and partially the Phlegrean Fields volcanic area. It includes a large part of the agricultural land belonging to more than 77 municipalities in the Naples and Caserta provinces (Capra *et al*., 2014) formerly identified by the Italian State as one of 54 National Interest Priority Sites (NIPS), where severe environmental pollution and degradation is supposed to occur and, therefore, where characterization and remediation activities have to be implemented. From January 2014, the area has been recognized as a Regional Interest Priority Site (RIPS) remitting any requalification activity to Campania Region.

Soils in the area are characterized by the presence of both detrital-alluvial sediments and the fall of pyroclastic material from Phlegrean Fields. Soil formation in the valley is strongly influenced by alluvial processes forming very thick, fertile soils which show moderate to high vertic properties (Capra *et al.*, 2014). The slopes are between 1-5% and the elevation ranges from 42 to 150 m above

sea level. The area has a Mediterranean climate, with an average annual temperature of 18.7 °C and an average annual rainfall of 818 mm (Capra *et al.*, 2014). In the Litorale Domitio Agro Aversano, agriculture is very intensive, mainly consisting of fodder crops (mostly maize and alfalfa), field horticulture, orchards and Buffalo live stock. It is well known the production of the famous "Mozzarella di Bufala Campana D.O.P." (Denomination Origin Protected) recognized with the Ministerial Decree on May 10, 1993.

Ancient Romans used to call this region *Campania felix* because of the land's fertility. Now this area is depicted by the media as an open-sky landfill. In 2004, the territory comprising the municipalities of Acerra, Nola and Marigliano, was named the "triangle of death" by the medical magazine Lancet due to its high incidence of cancer-related deaths and shorter lifespan of people living in this area. More recently, its moniker was changed to "land of fire", a reference to the common practice of burning rubbish in the area.

The area is actually characterized by a diffuse land abuse mainly through an intense and chaotic urbanization with most of municipalities reaching a population density higher than 425 inhabitants km^{-2} (ISTAT, 2013). Furthermore, the intensive agriculture and livestock, the presence of numerous dumping sites (both legal and illegal), the usual practice of waste incineration and the common sewage network leakage, has produced groundwater and soil contamination, with many wells showing very high nitrate concentration (Corniello and Ducci, 2014) and soil pollution by PTMs, hydrocarbons and pesticides (Capra *et al*., 2014; Bove *et al*., 2011; Grezzi *et al*., 2011).

This situation has driven Italian Government to institute a task force aimed at mapping pollution levels in agricultural soils of Campania. The task force, using pre-existing data and a soil sampling method based on composite soil samples taken on a 100 x 100 m grid pattern ,confirm until now only 22 ha of polluted soils within an agricultural area of 50.000 ha. The results of this survey were published on February 12th and July 7th, 2015 with two Interministerial decrees (GU n.56, 9-3-2015 and GU n.191, 19-08-2015). Nevertheless, the soil sampling scheme used in this characterization could lead to a significant underestimation of the risk posed by contaminants.

Authoritative researchers have recently suggested that Campania region could be a perfect field study for a monitoring research programme, as their poisoned fields could serve as a giant experiment in the new science of 'exposomics' (Nature, 2014). In three pilot sites of the Litorale Domizio Agro Aversano RIPS, subjected to unauthorized waste disposal, the EU-LIFE-Ecoremed 2011 (www.ecoremed.it) project is working to implement eco-compatible protocols for agricultural soil remediation.

Specifically, a 1000 m^2 rural area in the municipality of Giugliano (GI), a 4500 m^2 area in Trentola-Ducenta (TD) and a 3000 m^2 area in Teverola (TE).

2.2 Soil sampling

A soil sampling scheme based on a two-level grid resolution was defined. On the first level, each study area was divided into regular low-resolution 10x10 m grids (plots). On the second level, each grid plot was subdivided into nine high-resolution 3x3 m subplots, so that, at the finer resolution level, each soil sampling point is about 3.3 m away from the next. Discrete soil samples (DS) were taken from each 3x3 m subplot. Composite soil samples (CS) were made bulking aliquots (about 200g) from the previously homogenised nine DS taken from each 10x10 m plot. Soil samples were collected from both 'topsoil' (0–30cm depth) and 'subsoil'(30–60 cm depth) layers to evaluate vertical gradients. An outline of the sampling scheme, at the two levels, is given in Figure 1.

In total were identified: in Giugliano, 8 grids/CS and 72 DS; in Trentola-Ducenta, 28 grids/CS and 252 DS; in Teverola, 12 grids/CS and 108 DS. A total of 960 soil samples were collected of which 96 grids/CS and 864 DS. All samples was georeferenced. After preliminary analysis on 10x10 m CS (main soil physical and chemical properties, pseudototal PTM content), ten 10x10 m grids (4 in Giugliano, 4 in Trentola-Ducenta, 2 in Teverola) were selected for detailed analyzes on single 3x3 m DS.

2.3 Soil analysis

Soil samples were air-dried and sieved at 2 mm according with the official procedures published by Ministero delle Politiche Agricole e Forestali (MiPAAF,2000) prior to the determination of moisture content at 105 °C, pH–H_2O (1:2.5 soil:water ratio), organic carbon (Walkley and Black method, 1934), cation exchange capacity (CEC) (BaCl2 + TEA at pH 8.1) and carbonates (pressure Dietrich–Fruehling calcimeter method, Loeppert and Suarez, 1996). Particle size analysis (Andreasen's pipette method) was carried out by wet sieving and by sedimentation in aqueous media after organic matter oxidation by H_2O_2 treatment.

Pseudototal content of 16 PTMs defined as priority pollutants by Italian legislation (DL gs 152/2006) was measured. For 15 PTMs, microwave assisted acid digestion in *aqua regia* (EPA 3051A/2007) was used and concentrations in the digestion solution were determinate by inductively coupled plasma-atomic emission spectrometry Perkin Elmer ICP-AES Optima 7300DV (EPA 6020A/2007). For Hg, thermal decomposition, catalytic conversion, amalgamation and atomic absorptions spectrophotometry DMA-80 Mileston Inc. (EPA 7473/2007) was used.

2.4 Data analysis

For the purpose of this study, univariate statistical analysis was performed on the top and bottom CS and DS in order to show the single element geochemical distribution (Tables 1 and 2).

The degree of soil enrichment with PTMs was assessed calculating the following indices: contamination factor (C^i_f), contamination degree (C_{deg}), pollution load degree (PLI) and geoaccumulation index (I_{geo}) (Hakanson, 1980; Müller *et al*., 1981;Loska*et al*., 2004).The C^i_f is a single element index and is measured by the following equation 1 (Hakanson, 1980)

Eq. 1 $C^i_f = C^i_{0\sim1} / C^i_n$

where $C^i_{0\sim1}$ is the mean content of metals from sampling sites and C^i_n is the preindustrial concentration of individual metal. In this study, the background value of individual metal in soil from the study area (Lima *et al.*, 2014) was applied as preindustrial concentration. Values of the contamination factor are characterized as follow: $C^i_f<1$ indicating a low contamination of the soil by the considered metal, $1 \leq C^i_f< 3$ a moderate contamination, $3 \leq C^i_f< 6$ a considerable contamination, $6 \leq C^i_f$ a very high contamination. The sum of the C^i_f of all metals gives the overall C_{deg} of the soil environment ($C_{deg}< 8$, low degree of contamination; $8 \leq C_{deg}< 16$, moderate degree of contamination; $16 \leq C_{deg}< 32$, considerable degree of contamination, $32 \leq C_{deg}$, very high degree of contamination). As well, PLI, calculated by the equation 2

Eq. 2 $PLI = \sqrt[n]{Cif1 \; x Cif2 \; x \ldots\ldots Cifn}$

where n is the number of the metals, indicates if the soil is polluted (PLI >1) or unpolluted (PLI <1).

The I_{geo} is another single element index. It enables the assessment of environmental contamination by comparing differences between current and preindustrial concentrations and is measured by the equation 3

Eq. 3 $I_{geo} = \log_2 (C_n/1.5\, B_n)$

Where C_n is the metal concentration in soil, B_n is the metal geochemical background value and the 1.5 factor accounts for natural fluctuations in the content of a given metal in the soil environment also due to very small anthropogenic influences (Loska *et al*., 2004). According to Müller *et al*., 1981, the I_{geo} interpretation is: class 0, practically uncontaminated ($I_{geo} \leq 0$), class 1,

uncontaminated to moderately contaminated (0 $<I_{geo}<$ 1), class 2, moderately contaminated (1 $<I_{geo}<$2), class 3, moderately to heavily contaminated (2 $<I_{geo}<$3), class 4, heavily contaminated (3 $<I_{geo}<$4), class 5, heavily to very heavily contaminated (4 $<I_{geo}<$5) and class 6, very heavily contaminated ($I_{geo} \geq 5$).

3. Results and discussion

3.1 Soil properties

Soil physico-chemical properties were analyzed both on top (0-30) and subsurface (30-60) CS. Since no significant differences were observed with depth, only topsoil values are shown below. GI, TD and TE soils were medium to coarse textured sandy loam soils with 55±5.1% in TE, 61±2.5% in GI and 71±2.9% in TD of the soil in the sand fraction. In TE 14±2.6% of the soil was made by clay particles, while a lower content of clay particles characterises GI and TD (8±1.1 and 7±1.6% respectively).

The soil pH-H_2O was neutral in GI (pH 7.3±0.2) and sub-alkaline in TD (pH 8.0±0.2) and TE (pH 8.1±0.0). This is in agreement with the carbonate content higher in TD (174±41g kg^{-1}) and TE (92±1.4g kg^{-1}) than in GI (8±2.5g kg^{-1}). The soil pH-KCl was always lower than the soil pH-H_2O (GI 6.5±0.3, TD 7.3±0.2, TE 7.1±0.1) revealing the reserve acidity of soil colloids.

Electrical conductivity was 0.16±0.03 dSm^{-1} in GI, 0.45±0.3 dSm^{-1} in TD and 0.17±0.02dSm^{-1} in TE, indicating a general absence of salinity stress to plant growth and agricultural production.

Soil organic carbon content was always below 2%, with similar values in the three pilot sites (GI 20±3.1g kg^{-1}, TE 10±0.1g kg-1, TD 18±0.7g kg^{-1}). CEC ranges between 28±3.1 $cmol_{(+)}kg^{-1}$ in TD and 34±3.2 $cmol_{(+)}$ kg^{-1} in GI, with a dominance (~80%) of calcium on the exchange complex.

3.2 PTMs content in the composite soil samples

The descriptive statistics of 12 PTMs 'pseudototal' concentrations in the CS collected at the studied pilot sites are shown in Table 1.Selenium, Tl and Hg values are not reported because always below respective detection limits (0.5, 0.1 and 0.01 mg kg^{-1}). The table reports, for each element considered, the Italian Action Levels for Residential land use (IALR) established under Italian environmental law (D.Lgs 152/2006) and the local Soil Background Reference values for Be and V (ISPRA, 2010; Lima *et al*., 2014). In Italy soil quality standards for agricultural areas have not been established yet.

Beryllium, Cu, V and Zn were the only elements occurring in several soil samples in amounts above Italian legal limits. Beryllium and V exceed the Italian limits of 2 and 90 mg kg^{-1} in all surface and

subsurface soil samples. Nevertheless, when we compare the Be and V concentrations in the soils of the study pilot sites with the Soil Background Reference values of 6 and 150 mg kg^{-1} defined by ISPRA in collaboration with the Campania Region Environmental Agency (ISPRA, 2010), a very small number of samples are (slightly) anomalous, suggesting a diffuse geogenic origin for the elements in the studied soils. This peculiarity highlights the inadequacy of a unique reference value for each PTM for the whole of Italy. Due to the Italy's complex geological setting, legal limits should be established at a regional or sub-regional level especially in areas where volcanic and sedimentary environments co-exist, thereby influencing the soil geochemistry (Cicchella *et al*., 2005).

Copper exceeds the corresponding legal limit (120 mg kg^{-1}) in GI at three plots in the surface soil and at four plots in the sub-soil and in TD at one plot in both surface and subsurface soil. The mean Cu concentrations were 131 in surface and 124 mg kg^{-1} in subsurface soil samples from GI and 95 in top and 109 mg kg^{-1} in subsurface samples from TD. For TE, the mean level was 37 and 39 mg kg^{-1} in top and subsurface respectively, with no samples showing Cu concentration above the maximum allowed limit.

Zinc exceeds the corresponding legal limit (150 mg kg^{-1}) in GI in one surface soil sample and in TD in two plots at both depths. The mean concentration values for GI were 95 and 82 mg kg^{-1} in top and subsurface samples respectively while in TD values were 114 and 96 mg kg^{-1}. In TE no samples showed Zn concentration above the maximum allowed limit.

In GI, the mean values of all analyzed metals do not differ significantly in top and subsurface samples. Nevertheless, comparing the maximum values, some metals, like Sb, Cr, Ni, Pb, Cu and Zn were higher in top than in subsurface. Probably, in GI site the contaminants arrived from agricultural treatments in neighbouring fields (Cu, Zn) and from wastes (Sb, Ni, Pb) deposited on the soil surface.

Opposite situation occurs in TD and TE sites, where the maximum value of metals like Cu, Zn and Pb (only in TE) was higher in subsurface than in the top soil samples, suggesting waste burying in these sites.

In GI, the variability of the pseudototal concentration of As, Cr, Ni, Pb, Cu and Zn was higher in surface than in subsurface soil samples, the opposite was true for Sb and equal coefficients of variation was observed for Be, Co and V. Coefficients of variation below 10% were calculated for Be, Co, V and Ni (and As) (only in subsurface), indicating homogeneous distribution and a geogenic origin of these metals in soil. For all the other elements coefficients of variation were above 10% and up to 87% irrespective to the fact that concentrations were below the legal limits. Intense anthropogenic activity, such as excavation, disposal and mixing of soil matrix with

extraneous materials (wastes) as well as agricultural practices (Cu based pesticides) in the site likely increased heterogeneity of the surface soil.

Opposite situation occurred in TD and TE where the variability of pseudototal PTMs content was generally higher in subsurface than in the surface soil. In particular, in TD the coefficients of variation of As, Be, Cd, Co, Cr, Ni, Pb, Cu, V and Zn were higher in subsurface than in the surface. Equal coefficient of variation was observed for Sb. In TE, the coefficients of variation of Sb, Cd, Co, Cr, Ni, Pb, V and Zn were higher in subsurface than in the top. Equal CV was present for As, Be, Cu. The vertical trend of the coefficient of variation in this two sites, also when the concentrations of PTMs were below the legal limits, suggests that the metal occurrence in soil is likely affected by waste burying. A similar vertical trend was observed in the same pilot sites for organic pollutants by Monaco *et al*. (2015). Accordingly, for the majority of the analysed elements, the distribution of the concentration values was positively skewed or skewed to the right. This indicates that the mass of the concentration values was distributed around the mean with only few values above the mean and that the mean was always greater than (to the right of) the median.

3.3 PTMs content in the discrete soil samples

As described in materials and methods, on selected 10x10 m plots tagged as 'polluted' and not polluted, i.e. their topsoil or subsoil CS value was respectively lower and greater than 90% of the Italian legal limit for at least one metal, analyses were made at a nesting ratio of 1:3, i.e. at 3×3 m regularly spaced points for each plot. In table 2, data for two polluted (GI1 and GI2) and two not polluted (GI6 and GI8) plots out of eight of the Giugliano site are given. These discrete soil samples gave us the possibility to estimate the intraplot horizontal and vertical variance of PTMs content at a small-scale spatial resolution.

In table 2, the pseudototal PTMs contents of CS from the 4 selected 10x10 m plots were compared to the mean content, range (min and max) and coefficient of variation of the nine 3x3 m DS. In general, at both depths, the mean PTMs content of DS were higher than the values of the corresponding CS, highlighting soil pollution not detectable merely on the base of the CS values. For example, in GI1 plot (0-30 cm), the mean DS contents of Sb, Cu and Zn (5.68, 481 and 304 mg kg^{-1}, respectively) were twofold the contents of the corresponding CS (2.51, 276 and 174 mg kg^{-1}, respectively), indicating a Sb pollution of the soil other than the already known Cu and Zn pollution. The discrepancy between DS and CS contents was even more prominent when we compared max DS values with CS contents, indicating a high heterogeneity and spatial variability in metal content of DS. The GI6 plot (0-30 cm), which on the base of the CS metal contents was considered an unpolluted plot, appeared to contain max DS amounts of Cu and Zn (262 and 160 mg

kg^{-1}) higher than the max CS amounts (Cu 114 and Zn 82.9 mg kg^{-1}) and of Italian legal limits (Cu 150 and Zn 150 mg kg^{-1}). The soil heterogeneity also stems from the high CV of most of the analyzed elements (generally greater than 100% for Sb, Cd, Cu and Zn), with exceptions for As, Be and V of known geogenic origin. These results demonstrate that a large scale of sampling (i.e. 10x10m) cannot reflect properly the actual soil pollution. This is better shown in Figure 1, where metal content in soil established by CS analysis is graphically compared to the content established by DS analysis. Short range spatial resolution data (i.e. DS values) reveals in GI1 plot unforeseen Cu and Zn pollution foci with values well above the legal limits.

Taking into consideration the metal ranges (minimum and maximum values), it appeared that metal contents in surface samples were generally higher than in subsurface and this was even more evident in GI1 and GI2 polluted plots. This trend of contamination with depth observed for both CS and DS plots was a univocal indication that soil metal pollution did not arise from polluted waste burying but rather from waste dumping on surface.

3.4 Assessment of soil contamination at low and high resolution level

The degree of soil contamination, assessed calculating various indices (see M&M) on CS, is given in table 3 for the metals occurring in soil in concentration above local background or legal limits.

In GI site, on the base of the mean C^i_f values, a considerable contamination of the soil (both on surface and subsurface) emerged only for Cu ($C^i_f > 3$). Moderate or low is the contamination by As ($C^i_f \sim 1$), and by Sb, Pb and Zn ($C^i_f < 1$). In TD site, the C^i_f indicates moderate soil contamination by Sb, Pb, Cu and Zn, low contamination by As and Be, without differences between surface and subsurface. In TE, all metals except As ($C^i_f \sim 1$) occur in soil with a C^i_f value below 1, confirming a lack of soil contamination in the site. These outputs were supported by the overall C_{deg} and PLI indices which showed moderate soil contamination in GI and TD and low/absence of contamination in TE. As well, the I_{geo} values showed heavy and moderate soil contamination by Cu in GI and TD sites (classes 3 and 2 in surface and class 2 in subsurface), and no contamination in TE.

When the degree of soil contamination in GI site was assessed considering the PTMs content of the discrete soil samples, different outputs were obtained (table 4), indicating a soil contamination at very small scale (3x3 m), which did not appear at larger scale (10x10 m).

In particular in GI1A plot, on the base of the mean C^i_f values, a very high soil contamination by Cu occurred ($C^i_f > 14$). The contamination by Sb and Zn resulted considerable ($3 < C^i_f < 6$), and that by As moderate ($C^i_f \sim 1$). The metals occurred at high concentration in both surface and subsurface soil, with the former higher than the latter. A similar soil contamination degree was observed in GI2A and GI2B plots, characterized by a considerable Cu contamination ($C^i_f > 5$) and a moderate As and

Zn contamination ($1<C^i_f< 3$). In GI6 and GI8, unpolluted plots according to CS metal content, soil at both depth emerged to be from moderately to considerably contaminated by Cu (C^i_f~3in GI6; C^i_f~5 in GI8) and moderately contaminated by As and Zn (C^i_f~1).

Accordingly, on the base of C_{deg} index, GI1 showed a considerable overall degree of contamination in topsoil and a moderate one in subsurface (respectively 30 and 13). In GI2 and GI8, a moderate degree of contamination was present at both depths. In GI6, C_{deg} showed from low (in subsurface) to moderate (in topsoil) contamination.

The I_{geo} confirmed in GI1A and GI2A a contamination from moderate to extreme and indicated an heavy Sb, Cu and Zn contamination (class 4) in 'unpolluted' GI8-4A plot.

The differences in soil contamination by Sb, As, Cu and Zn, as assessed on CS and DS are showed graphically in Figure 1.

4. Conclusions

In this study, a two depths soil sampling scheme based on a two-level grid resolution, a regular 10x10 m low-resolution grid and a 3x3 m high resolution grid, was applied in three agricultural sites suspected of heavy metal pollution by waste disposal, dumping and incineration. This approach was chosen to investigate the spatial distribution patterns of pollutants in soil and to highlight the difficulties to assess appropriately the degree of soil pollution and the detection of pollution foci when large scale sampling is applied.

In the studied sites, we did not find any high and diffuse soil pollution, as expected. Only in one site (GI) the soil contained PTMs (Cu, Zn, Sb and As) well above Italian legal limits and local natural backgrounds. A large dishomogeneity in soil pollutant spatial distribution emerged from comparison of data taken at high and low resolution level. The degree of soil pollution, as assessed by pollution indices, worsen passing from 10x10 m to 3x3m scale of sampling. Pollution foci were detected only when soil was sampled at the 3x3 m scale. Unpolluted 10x10m plots were found to contain metals above legal limits when sampled at 3x3 m scale.

These results arise comprehensible doubts on the soil pollution monitoring based on a large scale sampling concerning the study area known as 'Land of Fires', where most of the soil data actually available have been taken at a large (1x1 km) or at a very large (5x5 km) scale. According to our results the uncertainty of these data might be very high, with potential underestimation of pollution degree and omission of pollution foci.

Appropriate soil sampling and pollution assessment are crucial for assessing soil volumes requiring remediation. In this study, for example, on the base of the 10x10 m data, only 40 and 15% of GI and

TD soil need to be cleaned up to a depth of 60 cm. These volumes would increase if calculation is done considering the 3x3 m scaled soil samples.

High resolution soil sampling is very expensive and arduous when monitoring soil contamination over large areas has to be done. An appraisal of the site history aimed to identify past and present potential contaminating activities and preliminary geophysical measurements might help to define the most appropriate sampling scheme. When a high spatial variability is suspected, it might be advisable to make a preliminary composite sampling at low-resolution, and only in the areas where the composite samples exceed the threshold/background values, proceed with an high-resolution sampling.

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Table 1. Descriptive statistics of PTM 'pseudototal' concentration (mg kg^{-1}) in the composite soil samples collected at the Giugliano, Trentola-Ducenta and Teverola pilot sites.

	Sb	As	Be	Cd	Co	Cr	Ni	Pb	Cu	V	Zn
Giugliano 0-30 cm (n=8)											
Mean	1.13	13.4	6.22	< 0.1	7.70	10.5	5.78	49.1	131	55.8	95.1
Median	0.91	13.8	6.15	< 0.1	7.58	8.27	5.35	40.0	115	55.7	83.2
Min	0.41	10.4	5.72	< 0.1	7.29	6.04	4.70	33.7	66.8	50.9	71.8
Max	2.51	14.5	7.14	< 0.1	8.53	22.7	8.25	96.8	275	59.5	174
CV*%	71	10	7	< 0.1	6	53	20	44	48	5	36
Skewness	1.14	-1.79	1.13	< 0.1	1.01	1.88	1.72	1.99	1.96	-0.66	2.26
Kurtosis	0.67	3.32	1.43	< 0.1	0.14	3.33	3.24	3.79	4.67	1.42	5.29
Giugliano 30-60 cm (n=8)											
Mean	0.61	14.1	6.51	< 0.1	7.73	7.93	5.28	38.4	124	56.6	82.6
Median	0.65	14.0	6.46	< 0.1	7.70	6.80	5.17	37.6	120	57.2	76.8
Min	0.03	12.7	5.72	< 0.1	7.34	5.65	4.67	31.5	68.4	52. 2	65.5
Max	1.51	15.9	7.42	< 0.1	8.37	14.3	6.29	52.9	205	60.5	112
CV*%	87	8	8	< 0.1	4	35	9	18	37	5	21
Skewness	0.87	0.17	0.30	< 0.1	0.89	2.10	1.27	1.41	0.50	-0.27	1.24
Kurtosis	1.19	-0.51	0.38	< 0.1	0.63	4.51	2.19	2.08	-0.26	-0.94	-0.13
Trentola-Ducenta 0-30 cm (n=28)											
Mean	1.37	11.7	4.80	0.27	6.73	15.6	5.92	52.7	95.3	48.7	114
Median	1.28	11.4	5.20	0.27	6.64	15.5	5.88	50.8	95.0	47.7	116
Min	0.15	9.05	1.96	0.09	3.95	9.67	3.61	30.3	63.7	30.5	73.9
Max	2.63	15.9	7.90	0.50	8.86	24.6	7.54	88.9	137	64.5	160
CV*%	55	15	33	38	17	26	15	29	18	15	21
Skewness	0.18	0.77	-0.16	0.51	-0.01	0.34	-0.35	0.72	0.44	0.23	0.07
Kurtosis	-1.10	0.28	-0.90	-0.03	0.20	-0.70	0.84	-0.15	0.59	0.97	-0.85
Trentola-Ducenta 30-60 cm (n=28)											
Mean	1.47	12.0	5.06	0.24	6.82	14.4	6.47	45.0	109	49.1	96.3
Median	1.43	11.6	5.29	0.23	7.25	13.4	6.46	42.0	89.4	50.4	89.2
Min	0.36	6.82	1.49	0.10	3.04	7.07	2.87	18.1	40.9	26.6	43.7
Max	3.46	18.0	8.56	0.57	9.93	26.9	20.1	82.1	747	67.6	161
CV*%	52	24	38	42	27	37	46	35	116	22	31
Skewness	0.87	0.50	-0.02	1.58	-0.22	0.85	3.60	0.62	5.01	-0.28	0.40
Kurtosis	0.86	-0.55	-0.72	4.04	-0.74	0.20	16.9	0.09	25.9	-0.74	-0.38
Teverola 0-30 cm (n=14)											
Mean	0.87	11.7	5.36	0.19	6.12	16.8	6.50	34.2	37.0	51.3	60.1
Median	0.86	11.4	5.33	0.19	5.96	17.1	6.63	34.4	38.5	50.1	59.4
Min	0.47	9.93	4.20	0.10	4.77	12.9	5.48	26.4	22.3	41.7	43.1
Max	1.62	15.9	6.88	0.25	7.67	20.6	7.58	44.5	44.4	62.6	77.6
CV*%	36	14	17	23	16	14	10	15	16	14	16
Skewness	0.91	1.29	0.21	-0.47	0.20	-0.05	-0.08	0.35	-1.34	0.15	0.07
Kurtosis	0.82	1.95	-1.27	-0.40	-1.37	-0.87	-1.51	-0.43	2.04	-1.36	-0.23
Teverola 30-60 cm (n=10)											
Mean	0.77	11.6	5.35	0.30	6.07	17.1	6.42	38.2	39.1	50.9	61.9
Median	0.82	11.3	5.08	0.19	5.89	17.8	6.57	34.8	37.7	49.9	59.4
Min	0.20	9.48	4.14	0.14	4.59	13.0	5.21	27.6	32. 0	40.8	44.2
Max	1.34	15.0	6.79	1.16	7.89	20.3	7.49	71.8	48.8	65.8	82.6
CV*%	47	14	18	105	17	17	12	34	15	15	20
Skewness	-0.19	0.90	0.41	3.02	0.37	-0.36	-0.10	2.25	0.89	0.64	0.53
Kurtosis	-0.86	0.85	-1.33	9.33	-0.64	-1.68	-1.36	5.81	-0.20	-0.21	-0.40
Italian limit**	**10**	**20**	**2 (6)**	**2**	**20**	**150**	**120**	**100**	**120**	**90(150)**	**150**

* Coef.Var.= CV

**D. Lgs. 152/2006; in brackets the local Soil Background Reference values (ISPRA. 2010)

Table 2. Descriptive statistics of PTM 'pseudototal' concentration (mg kg^{-1}) in the discrete soil samples collected at the Giugliano (GI) pilot site.

	Sb	As	Be	Cd	Co	Cr	Ni	Pb	Cu	V	Zn
GI plot 1- 0-30 cm											
CS*	2.51	10.4	5.72	0.46	7.30	14.6	8.25	44.5	276	50.9	174
Mean DS*	5.68	14.7	6.48	0.93	8.26	25.4	9.61	60.3	481	54.7	304
Min	<0.5	12.4	5.52	<0.5	7.59	7.55	4.90	44.5	110	47.0	92.8
Max	14.9	16.9	7.28	3.19	9.67	77.9	28.7	87.2	1707	58.8	972
CV*%	85	10	11	145	10	98	85	27	109	7	103
GI plot 1- 30-60 cm											
CS*	0.64	12.8	6.84	<0.5	7.69	9.50	5.63	38.1	163	58.0	109
Mean DS*	1.85	13.6	5.59	0.08	6.86	11.6	5.99	45.6	200	55.4	135
Min	<0.5	12.8	4.99	<0.5	6.17	5.71	4.42	31.3	76.8	50.0	73.5
Max	4.73	15.6	6.56	0.48	7.40	20.4	7.77	65.8	469	63.2	246
CV*%	82	7	9	217	5	41	19	22	55	7	45
GI plot 2- 0-30 cm											
CS*	1.62	13.4	7.14	<0.5	8.53	22.7	6.03	38.1	152	56.2	110
Mean DS*	3.09	18.3	6.02	0.89	9.26	17.9	6.79	47.5	191	55.5	131
Min	<0.5	16.4	5.72	<0.5	7.08	7.23	4.96	32.4	84.1	51.9	73.5
Max	11.5	20.3	6.53	5.72	19.6	35.5	10.9	77.0	429	60.5	225
CV*%	125	8	5	209	43	66	29	30	68	5	50
GI plot 2- 30-60 cm											
CS*	<0.5	13.7	4.92	<0.5	7.43	9.30	4.85	45.2	158	54.5	111
Mean DS*	<0.5	14.3	5.84	0.08	8.31	12.2	6.79	46.1	196	57.7	136
Min	<0.5	12.8	5.20	<0.5	7.51	7.25	4.84	37.9	105	53.6	80.7
Max	<0.5	15.5	6.71	0.74	12.3	20.4	11.4	60.4	580	64.2	342
CV*%	-	5	9	300	18	41	28	18	76	5	63
GI plot 6- 0-30 cm											
CS*	0.04	14.2	6.25	<0.5	7.32	9.57	4.70	38.0	114	55.2	82.9
Mean DS*	0.34	14.5	6.98	<0.5	7.35	7.63	4.71	39.4	105	55.9	83.1
Min	<0.5	12.6	6.23	<0.5	6.27	4.32	3.29	27.7	33.0	51.4	55.8
Max	1.57	16.0	7.73	<0.5	8.16	13.3	7.85	61.5	262	61.8	160
CV*%	199	7	7	-	9	34	28	26	66	6	37
GI plot 6- 30-60 cm											
CS*	<0.5	10.9	4.27	<0.5	7.40	6.19	4.75	35.3	68.7	64.2	75.9
Mean DS*	0.14	14.4	6.01	<0.5	7.42	7.36	5.60	37.2	86.1	56.2	78.0
Min	<0.5	13.4	5.35	<0.5	6.71	6.54	4.79	32.5	61.8	53.7	71.6
Max	1.23	15.4	6.67	<0.5	8.38	7.88	7.00	40.9	130	60.2	93.0
CV*%	300	4	6	-	7	6	13	6	29	4	9
GI plot 8- 0-30 cm											
CS*	0.41	14.3	5.79	<0.5	7.54	6.04	5.52	33.7	66.8	55.3	72.7
Mean DS*	0.95	16.7	7.04	0.11	8.71	9.24	6.11	46.7	169	56.0	156
Min	<0.5	14.0	6.22	<0.5	7.10	4.46	3.76	29.3	32.6	50.7	58.4
Max	6.39	29.6	7.62	1.01	16.8	29.0	13.3	99.9	859	59.4	719
CV*%	228	29	7	300	35	83	50	45	155	5	136
GI plot 8- 30-60 cm											
CS*	<0.5	12.7	3.20	<0.5	7.26	7.15	4.63	41.3	87.0	54.9	95.2
Mean DS*	0.53	14.7	5.98	0.60	9.33	8.57	6.14	42.1	107	57.1	125
Min	<0.5	13.0	5.38	<0.5	7.34	5.48	4.80	34.0	46.8	52.3	63.9
Max	2.44	20.3	6.59	1.19	13.0	18.9	8.11	64.0	297	61.8	485
CV*%	198	16	8	78	19	48	14	23	73	6	109
Italian limit**	**10**	**20**	**2 (6)**	**2**	**20**	**150**	**120**	**100**	**120**	**90(150)**	**150**

*Composite sample=CS; Discrete sample =DS; Coef.Var.= CV

**D. Lgs. 152/2006; in brackets the local Soil Background Reference values (ISPRA. 2010)

Table 3. C^{i}_{f}, C_{deg} and PLI in the composite soil samples collected at the Giugliano (GI), Trentola-Ducenta (TD) and Teverola (TE) pilot sites.

Sample/Depth	C^i_f -Sb	C^i_f -As	C^i_f -Pb	C^i_f -Cu	C^i_f -Zn	C_{deg}	PLI
GI 0-30 cm	0.79	1.31	0.70	3.75	0.95	9.48	1.14
GI 30-60 cm	0.43	1.28	0.55	3.55	0.83	8.68	1.00
TD 0-30 cm	1.27	0.81	1.17	2.71	1.14	8.93	1.17
TD 30-60 cm	1.03	0.83	1.10	2.50	0.97	8.25	1.10
TE 0-30 cm	0.81	1.01	0.64	0.70	0.73	5.46	0.77
TE 30-60 cm	0.72	1.00	0.71	0.74	0.76	5.50	0.78

Table 4. C^{i}_{f}, C_{deg} and PLI in the discrete soil samples, in selected plot, collected at the Giugliano (GI) site

Sample/Depth	C^{i}_{f} -Sb	C^{i}_{f} -As	C^{i}_{f} -Pb	C^{i}_{f} -Cu	C^{i}_{f} -Zn	C_{deg}	PLI
GI1 0- 30 cm	5.46	1.32	0.88	14.9	3.32	30.1	2.70
GI1 30-60 cm	1.72	1.24	0.65	5.72	1.35	13.0	1.45
GI2 0- 30 cm	2.88	1.66	0.68	5.46	1.31	14.7	1.69
GI2 30-60 cm	0.00	1.30	0.66	5.59	1.36	10.9	0.00
GI6 0- 30 cm	0.32	1.32	0.56	3.00	0.83	8.06	0.93
GI6 30-60 cm	0.13	1.31	0.53	2.46	0.78	7.00	0.75
GI8 0- 30 cm	0.88	1.52	0.67	4.83	1.56	11.6	1.33
GI8 30-60 cm	0.49	1.34	0.60	3.05	1.25	8.89	1.08

* topsoil = 0- 30 cm depth; Subsurface = 30-60 cm depth

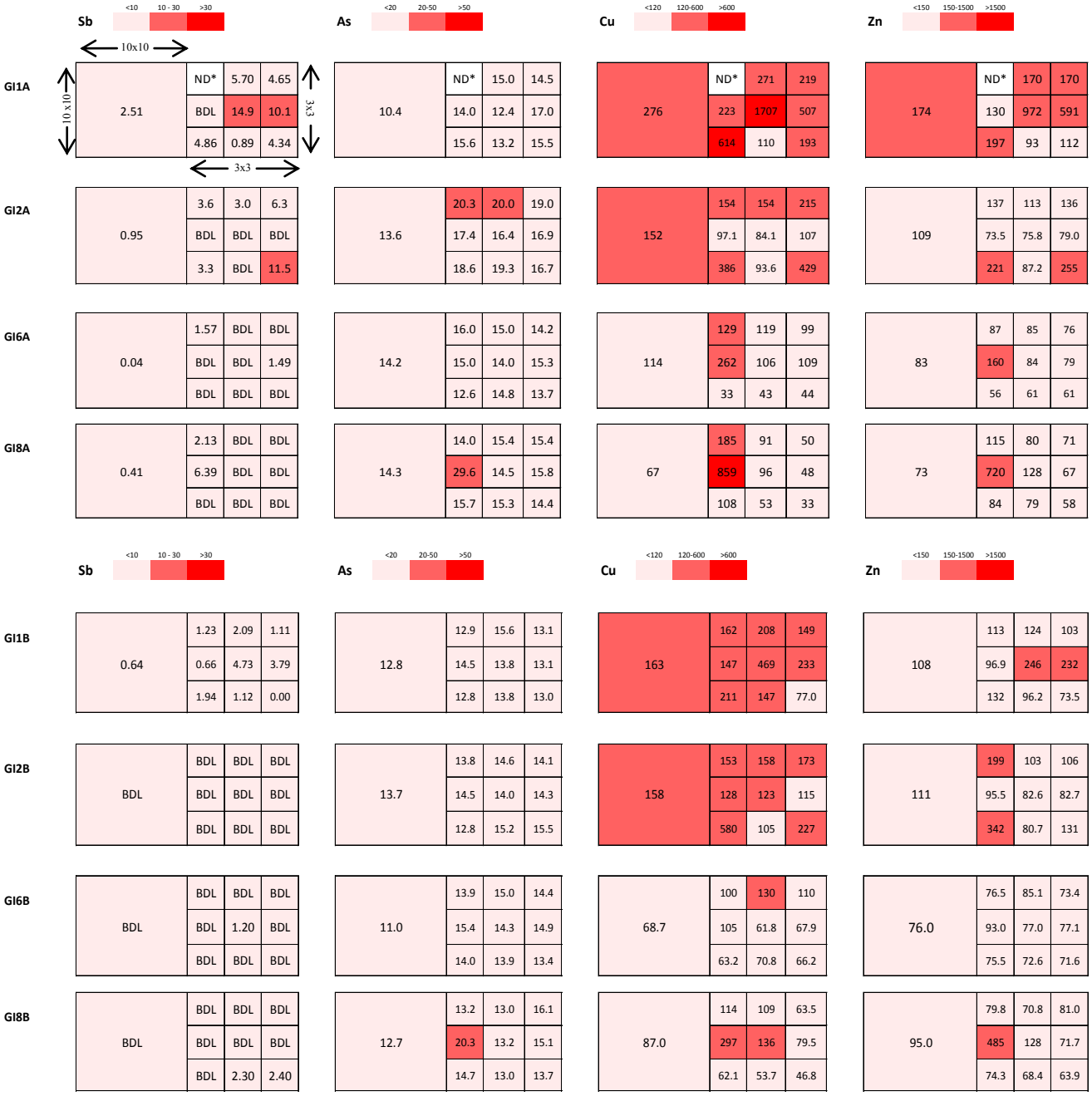

Figure 1. Graphical representation of PTMs content (mg kg^{-1}) in four selected 10x10 m and 9 corresponding 3x3 m plots of Giugliano (GI) site (A = 0- 30 cm; B = 30-60 cm).
*Not determined

CHAPTER II

Chemical extraction procedures to predict potentially toxic metals mobility and bioavailability in phytoremediation treatments

1. Introduction

Potentially toxic metals (PTMs) are usually present in soil as a consequence of natural weathering process or anthropogenic input. The chemical behaviour of these metals depends on the soil properties (pH, soil texture, organic matter and mineral composing), chemical (metals may be adsorbed on clay surface, bound with carbonates, organic matter or Fe oxyhydroxides) and biological processes (due to changes of physic-chemical condition from plants root or microbiological activities) (Leleyter *et al.*, 2012; Temminghoff *et al.*, 1997).

PTMs are persistent in soils for a long time after their introduction because of the retention ability of soils and they are recalcitrant to biodegradation, therefore PTMs pollution can only be remediate by their removal from soil (Bolan *et al.*, 2014). With this aim, several authors (Bolan *et al.*, 2014; Megharaj *et al.*, 2011; Van Nevel *et al.*, 2007; Keller and Hammer, 2004; Raskin *et al.*, 1997) have studied 'green' techniques to recovery polluted soils. Among these techniques is the phytoremediation, defined by Raskin *et al.* (1997) as the use of green plants to remove or reduce pollutants from the environment or render them harmless. This technology is based on the concept of mobility and bioavailability of metals. The bioavailability, more than the total PTMs content in soil, exerts decisive impact on soil quality and its use in food production. Thus, during the last decades, the accurate estimation of soil PTMs bioavailability is becoming an increasing concern as the risk assessment based on the sole PTMs total content can lead to misleading results (Adamo and Zampella, 2008; Menzies *et al.*, 2007; Keller and Hammer, 2004). Bioavailability is defined as the capacity of an element to be transferred from a soil fraction to a living organism, regardless of mechanism (Leleyter *et al.*, 2012).

A rapid assessment of metals bioavailability is generally done by using several single chemical extractants. Among them, there are unbuffered salt solutions as NH_4NO_3, a suitable method for the determination of readily soluble and plant available trace element contents (Gryshko *et al.*, 2005), and chelating agents as EDTA, with high extraction capacity able to displace metals from insoluble organic or organometallic complexes (Rauret *et al.*, 1998). These two extractants are frequently applied because of their ability to precipitate most of the

colloids and part of soluble metal-organic complex in 1M NH_4NO_3 (Gryshko *et al.*, 2005) and form very stable complex with a wide range of cations in 0.05M EDTA (Rauret *et al.*, 1998). Generally, the fraction extracted by NH_4NO_3 is recognized as the plant available fraction (Gupta and Aten, 1993; Gryshko *et al.*, 2005) fitting well for metals which are mainly bound by low strength electrostatic forces to the soil.

In addition to the single chemical extractants, sequential extraction procedures are carried out with selective extractants of increasing strength to study the origin, the fate and biological availability, potential mobility and transport of PTMs in environment (Leleyter *et al.*, 2012; Nemati *et al.*, 2009).

The main aim of this work was to assess the mobility and bioavailability of Cu and Zn in agricultural polluted soils in order to predict the feasibility and effectiveness of phytoremediation treatments. For this purpose, single and sequential chemical extractions were applied in a complementary approach. Bioavailability as predicted by chemical extractions was compared with effective Cu and Zn plant uptake. The effect of phytoremediation treatments on Cu and Zn bioavailability in soil was investigated after one year from planting.

2. Materials and Methods

2.1 Soil samples

Soil samples polluted by Cu and Zn were collected in two agricultural areas of southern Italy, Giugliano (GI) and Trentola Ducenta (TD), subjected to waste disposal, chosen as pilot sites of the LIFE-Ecoremed project (www.ecoremed.it). Details about sampling sites and methods are given in M&M section of chapter 1. This study was carried out on a selection of discrete soil samples chosen on the base of the total PTMs content.

2.2 Soil analysis and extractions procedures

Soil samples were air-dried and sieved at 2 mm according with the official procedures published by Ministero delle Politiche Agricole e Forestali (MiPAAF, 2000) prior to the determination of moisture content at 105 °C, pH–H_2O (1:2.5 soil:water ratio), electric conductivity (1:5 soil:water ratio), organic carbon (Walkley and Black method, 1934), cation exchange capacity (CEC) ($BaCl_2$ + TEA at pH 8.1) and carbonates (pressure Dietrich–Fruehling calcimeter method, Loeppert and Suarez, 1996) were analysed. Particle size analysis (Andreasen's pipette method) was carried out by wet sieving and by sedimentation in

aqueous media after organic matter oxidation by H_2O_2 treatment. The different forms of Fe, Al and Si in soil (< 2 mm) were analysed by the acid ammonium oxalate (Schwertmann, 1964) and dithionite–citrate–bicarbonate (Holmgren, 1967) dissolution techniques.

Pseudototal content of 16 PTMs defined as priority pollutants by Italian legislation (DL 152/2006) was measured. For 15 PTMs, microwave assisted acid digestion in aqua regia (EPA 3051A/2007) was used and concentrations in the digestion solution were determinate by inductively coupled plasma-atomic emission spectrometry Perkin Elmer ICP-AES Optima 7300DV (EPA6020A/2007). For Hg, thermal decomposition, catalytic conversion, amalgamation and atomic absorptions spectrophotometry DMA-80 Mileston Inc. (EPA 7473/2007) was used.

Bioavailable Cu and Zn pool in soil was determined by extraction with 2 several methods, 1M NH_4NO_3 solution (1:2.5 w/v soil:solution ratio) was added to the soils and put for two hours in an end-over-end shaker (DIN, 1995). The metals concentrations were analyzed with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS, Perkin Elmer), and 0.05M EDTA pH 7 (1:10 w/v soil:solution ratio) extraction was conducted using the method described by European commission (EUR 19774 EN/2001). Extractible metals concentration were analyzed with inductively coupled plasma-atomic emission spectrometry Perkin Elmer ICP-AES Optima 7300DV. These methods were applied two times, before and during - after one year - phytoremediation treatments, firstly to evaluate the possibility to applied with success phytoremediation, and then for measure how the bioavailability change during the treatments.

The four-step chemical extraction procedure (0.11M HOAc – Step 1, 0.1M NH_2OH HCl – Step 2, H_2O_2/1M NH_4OAc – Step 3, aqua regia – Residual), developed by the Measurement and Testing Programme of the European Commission, was used to fractionate Cu and Zn geochemical forms (Ure *et al.*, 1993) on the two most polluted subplots from Giugliano site (GI1-5 and GI1-6).

2.3 Phytoremediation treatments and plant analysis

In GI and TD sites, several combinations of compost (C) (derived by plant residual), *Trichoderma sp*. (T), microorganisms (Bio) and plants (P) were applied to 3x3 m plots within each experimental site. In particular, *Eucalyptus camedulensis* L. and *Populus nigra* L. were adopted for the assisted phytoremediation in GI and TD, respectively, because already used in other studies of phytoremediation for their high biomass and tolerance to metals (Luo *et al.*, 2015; Evangelou *et al.*, 2014). *Eucalyptus* is characterised by fast growth, extended root apparatus, high biomass production and notable adaptability to different pedo-climatic

conditions. Another advantage of using *Eucalyptus* is the low risk of PTMs entering in the food chain (Luo *et al.*, 2015; Shah *et al.*, 2011; King *et al.*, 2008). Deep ripper (0.40 -0.5 m depth) followed by rotary arrow (0.20 m depth) were used for soil preparation at both sites in order to reduce soil compaction. Trees were manually transplanted in march 2015 according to a density of 5000 plant ha^{-1}.

In this chapter soil results for both sites are presented. The data related to the plant uptake refer only to GI site.

After one year from transplanting, seven plots were selected matching treatments with soil pollution levels. Soil and plant leaves were collected and analyzed in order to assess metal uptake and the effect of phytoremediation on metal bioavailability. Soil was sampled following the same procedure used before transplanting. Plant leaves were collected from harvested aboveground plant parts. Leaves were washed with distillate water, oven-dried at 65 °C until constant weight and smashed for chemical analyses.

Leaves were chosen because they represent one of the major sink organ of the plant. Total content of metals in leaf tissues was measured using microwave assisted acid digestion in HNO_3 and concentrations in the digestion solution were determined by inductively coupled plasma-atomic emission spectrometry Perkin Elmer ICP-AES Optima 7300DV.

2.4 Data analysis

All data are presented as mean values with relative standard deviation of three replicates.

A correlation analysis was performed on data from soil single chemical extractions, before and after one year of plant growth, total metal content of leaves and total metal content of soil. Single and sequential extractions were compared with results from acid ammonium oxalate extractions and amounts of Cu and Zn in leaves (see Figure 1 and 2).

Uptake efficiency (Table 5) of plants in accumulating Cu and Zn into their tissues from soil was assessed using the accumulation factor (A) as described by Ali *et al.* (2013):

$$A(\%) = (C_{tissue}/C_{soil})x100$$

Where C_{tissue} is the metal concentration in plant harvested tissue and C_{soil} is the metal concentration in soil.

3. Results and discussion

3.1 Soil properties

The analyzed physico-chemical properties of soil samples are shown in Table 1. The soil pH-H_2O was neutral in GI (pH 7.3±0.2) and sub-alkaline in TD (pH 8.0±0.2). This is in agreement with the carbonate content higher in TD (174±41g kg^{-1}) than in GI (7.6±2.5g kg^{-1}). The soil pH-KCl was always lower than the soil pH-H_2O (GI 6.5±0.3, TD 7.3±0.2) revealing the reserve acidity of soil colloids.

Electrical conductivity was 0.16±0.03 dSm^{-1} in GI and 0.45±0.3 dSm^{-1} in TD, thus indicating that salinity is not limiting to plant growth and agricultural production. Soil organic carbon content was always below 20 g kg^{-1}, with similar values in the two pilot sites (GI 20±3.1 g kg^{-1}, TD 18±0.7 g kg^{-1}). CEC ranges between 28±3.1 $cmol_{(+)}kg^{-1}$ in TD and 34±3.2 $cmol_{(+)}\ kg^{-1}$ in GI, with a dominance (~80%) of calcium on the exchange complex.

The dissolution data in table 2 may be considered as indicators of allophanic materials and iron-rich phases (Dahlgren and Ugolini, 1991), which frequently represent a significant part of the colloidal fraction of soils from volcanic systems (Arnalds *et al.*, 2007). According to the extremely high Al/Si molar ratio the concentration of allophane is negligible in the studied soils. The Fe_o/Fe_d ratio indicates the presence of amorphous/non-crystalline iron oxides and, according to the formula proposed by Childs (1985) (Fe_{est} = 1.7 * Fe_o %), a moderate amount of ferrihydrite (0.54%).

3.2 PTMs extractability by single extractions and plant uptake

The amounts of Cu and Zn extracted by 1M NH_4NO_3 and 0.05M EDTA solutions from GI and TD discrete soil samples are shown in tables 3 and 4. As expected, the extractability of Cu and Zn by EDTA chelating agent was always higher than that of NH_4NO_3 unbuffered salt. Indeed, the amount of both metals extracted by 1M NH_4NO_3 was from 0.1 to 7.9 % of respective total contents while the amount extracted by 0.05 M EDTA ranged from 13 to 64 % of total content. EDTA is a strong chelating agent, can form stable chelates with many metal ions (Feng *et al.*, 2005) and can remove organically-bound metals, along with metals occluded in oxides and secondary clay minerals in part (Payà-Pérez *et al.*, 1993). EDTA is a non-specific extractant and can remove both labile and non-labile fractions (Bermond *et al.*, 1998). The 1M ammonium nitrate removes readily soluble and exchangeable metal forms (Gryschko *et al.*, 2005). In any case, results from single chemical extractions indicate

consistent amount of readily and potentially bioavailable Cu and Zn in studied polluted soils and therefore encourage the application of phytoremediation treatments.

Tested treatments did not affect Cu and Zn content of leaves. The content of both metals in leaves increased with total metal concentration in soil (R^2: 0.534 for Cu and 0.786 for Zn). The content of Zn in leaves was always higher than Cu (Cu: from 5.1 to 19.2 mg kg^{-1}; Zn: from 19.4 to 59.2 mg kg^{-1}). This is a common trend in plants as observed by Kabata-Pendias (2001) (Zn: 20-46 mg kg^{-1}; Cu: 2-11 mg kg^{-1}) and related to the plant physiology.

The uptake of Zn and Cu by *Eucalyptus ficifolia* grown on unpolluted soil from local field of Mannarguti (India) was studied by Priya and Jenifer (2014). In leaves the Cu and Zn concentrations were found to be 1.42 and 3.52 mg kg^{-1}, respectively. The higher amount of both metals uptaken by *Eucalyptus* plants grown on GI polluted soil (on average Cu 12.1mg kg^{-1}, Zn 39.3mg kg^{-1}) supported the attitude of *Eucalyptus* as a plant suitable for metal phytoremediation.

Plant uptake efficiency, as assessed by the leaves accumulation factor (A%) (Table 5), was found between 0.8- 18.3% for Cu and 2.8-41.4% for Zn, in accordance to higher Zn uptake by plants. When the metal concentration in soil was very high, a lower plant efficiency was observed despite of the larger amounts of metal extracted. Indeed, in GI1-5, the highest polluted point, the efficiency observed was around 1% for Cu and 3% for Zn, although the amounts of Cu and Zn uptaken by plant was around 19 and 34 mg kg^{-1}, respectively. On the contrary, in the unpolluted GI8-3 the efficiency was around 18 and 41% for Cu and Zn, with a plant uptake around 9 and 29 mg kg^{-1} of Cu and Zn. A similar observation was done by Wilson and Pyatt (2007) comparing Accumulation factors of Cu in olive plant tissues collected from a control site with those from a site adjacent to copper workings spoil. According to the Authors, it would appear that a higher proportion of the copper concentration in the control soil was available for bioaccumulation.

A correlation analysis was performed to investigate the relationship between chemically assessed bioavailable Cu and Zn amounts and total content of both metals in soil and in plant. As shown in table 6, both 1M NH_4NO_3 and 0.05 M EDTA extracted Cu and Zn amounts were positively and significantly ($P<0.05$) correlated with total Cu and Zn content in soil, indicating an increase of bioavailable amounts with total metal content in soil. When extracted amounts were expressed as percentage of the total content, no correlation with total content was observed, although lower percentages of both metals were extracted by EDTA from highly polluted soil samples. This might be presumably due to a reduced efficiency of

the 0.05M EDTA extractant solution when used at a ratio 1:10 w/v on highly polluted soil samples.

A positive correlation (P=0.10) was found between Cu and Zn amounts uptaken by plants and the Cu and Zn total content in soil. A trend, but not a significant correlation, was observed between the Cu and Zn bioavailable amounts assessed by single extractants before transplanting and the Cu and Zn content in plant leaves after one year of planting (Table 7). The comparison between metal bioavailable amounts in soil (as assessed chemically) and the metal amounts uptaken by plant is not easy. For a correct comparison we should calculate the whole plant uptake, while we collected and analysed only the leaves and not the whole plant, which were left to grow in the soil for a second year.

3.3 Sequential extractions and comparison with single extractions and plant uptake

The distribution of Cu and Zn between several sequentially extracted fractions in two differently polluted soil samples from Giugliano site (GI1-5, Cu 1707 mg kg^{-1}, Zn 972 mg kg^{-1}; GI1-6, Cu 507 mg kg^{-1}, Zn 591 mg kg^{-1}) is given in figures 1 and 2. Metals extracted by ammonium nitrate, EDTA, acid ammonium oxalate and the leaves content were used as references to interpret results of sequential extractions. Cu was mostly associated with reducible phases (54 and 55%, Step 2) and in a minor part with oxidizable phases (19 and 24%, Step 3). Zinc was equally distributed between HOAc-extractable (35 and 45%, Step 1) and reducible (29 and 43%, Step 2) fractions. The data obtained by sequential extractions indicate an higher mobility of Zn with respect to Cu, confirmed by the higher amounts of Zn extracted by leaves. Iron and aluminum oxides and organic matter are the most commonly involved solids in the adsorption of metals in soil. Copper, as described by Azevedo Silveira and Ferracciú Alleoni (2003), has a great affinity with functional groups of soil organic matter. The high amount of Cu released in step 2 (reducible fraction), suggests that not only electrostatic mechanisms are involved in adsorption, but mechanisms of specific adsorption are probably responsible for Cu retention in the studied soils. The occurrence of amorphous Fe oxides, other than organic matter, in the studied soil are likely responsible for Cu adsorption. This is indirectly confirmed by the consistent amounts of Cu released from the GI soil by oxalate extraction (250±10.5 mg kg^{-1}), comparable to the amounts of Cu extracted by EDTA (215±5.3 mg kg^{-1} in GI1-5 and 194±5.5 mg kg^{-1} in GI1-6) and by hydroxylamine (926±187 mg kg^{-1} in GI1-5 and 279±22.2 mg kg^{-1} in GI1-6). Furthermore, also the Fe amounts released by the step 2 of the sequential procedure (3015±3.8 mg kg^{-1} in GI1-5 and

2067±1.5 mg kg^{-1} in GI1-6) were comparable with the Fe amounts extracted by oxalate from the GI soil (2757±62.3 mg kg^{-1}).

For both soil samples, the 1M NH_4NO_3 solution extracted amounts of Cu similar to the amounts uptaken by plants, while an under estimation was observed for Zn.

EDTA played a significant role to extract potentially bioavailable metals in soil, showing an higher affinity for Zn than for Cu (about 40% and 56% of total Zn against about 13% and 38% of total Cu extracted by EDTA in GI1-5 and GI1-6, respectively).

3.4 Effect of phytoremediation treatments on bioavailability

In figures 3 and 4 the values of Cu and Zn extracted by NH_4NO_3 and EDTA solutions before and after one year of planting are compared. Not significant differences were observed among different treatments.

A general reduction of both NH_4NO_3 and EDTA bioavailable amounts of Cu and Zn was observed after planting regardless of the initial concentrations (NH_4NO_3: from 19 to 60% for Cu and from 43 to 92% for Zn; EDTA: from 4 to 27% for Cu and from 8 to 76% for Zn). When the initial concentration was lower than legal limit, the amounts of bioavailable fractions was not significantly different before and after phytoremediation. Despite the reduction observed after one year of planting, the bioavailable amounts of Cu were still above the trigger value of 1 mg kg^{-1} defined by German DIN for NH_4NO_3 procedure for the pollutant transition soil-food plant on agricultural areas. Differently, the bioavailable amounts of Zn, which before planting were above the German DIN trigger value of 2 mg kg^{-1}, go below that limit after one year of phytoremediation practice. This result might be related to the greater Zn mobility and bioavailability in soil, as assessed by single and sequential extractions, and to the higher Zn uptake by the plants, as shown by the higher amounts of Zn found in the plant leaves (Schwartz *et al.*, 2003).

4. Conclusions

Single and sequential extraction procedures were applied to study Cu and Zn bioavailability and fractioning among geochemical forms in agricultural polluted soils in order to assess phytoremediation feasibility and effectiveness. The readily and potential mobility and bioavailability of both metals, higher for Zn than for Cu, as assessed by the single and sequential chemical extractions, is a reliable indication of the suitability of plant cropping as soil remediation treatment. Indeed, after one year from *Eucalyptus camedulensis* planting, a

significant reduction of the bioavailable amounts of Cu and Zn in soil, as assessed by single chemical extractions, was observed in both sites without any significant difference between the applied treatments. A positive trend, but not a significant correlation was observed between Cu and Zn amounts extracted by NH_4NO_3 and EDTA, and the metals content in leaves. From the comparison between the Cu and Zn amounts extracted by NH_4NO_3 and those uptaken by plants (leaves content), a general underestimation of bioavailable Zn as assessed by chemical extractions was observed. A more detailed information comes from the sequential extractions. In the studied soils, according to sequential extractions, an higher mobility, and therefore bioavailability, characterizes Zn respect to Cu. Indeed, Zn was principally associated to the easily HOAc-extractable and reducible fractions (steps 1 and 2), while Cu was mostly found in reducible and oxidizable phases (steps 2 and 3). These results seem to be confirmed by Zn uptake by plant always higher than Cu. Thus, we would suggest for polluted soils to combine single and sequential chemical extractions in order to obtain a more accurate description of the chemical status of metals in soil and therefore a better prediction of their bioavailability.

Although our data are not definitive, because the plants are planned to grow on the polluted soils for a total of two years and only at the end of this period it will be possible to calculate the accurate removal of metal pollutants by plant, we observed a decrease of the plant uptake efficiency, assessed as leaves accumulation factor, in correspondence of highly polluted sub-plots. This is not surprising, due to the fact that *Eucalyptus* is not an hyperaccumulator plant. Nevertheless, in our case the removal of metals from the soil by *Eucalyptus* is an evidence and even if in a consistent number of years the chosen phytoremediation treatment should clean up the soil reintegrating it to the original agricultural use devoted to food production.

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Table1. Physico-chemical properties (mean ± SD, n= 10) of agricultural soil samples in Giugliano (GI) and Trentola Ducenta (TD) sites.

Site	**Texture (%)**			**pH**	**pH**	**CE**	**CO**	**Carbonates**	**CEC**
	Sand	Silt	Clay	H_2O	KCl	(dS m^{-1})	(g kg^{-1})	(g kg^{-1})	(cmol(+) kg^{-1})
GI	61	31	8	7.3±0.2	6.5±0.3	0.16±0.0	24.1±1.5	7.6±2.5	34±3.2
TD	71	22	7	8.0±0.2	7.3±0.2	0.45±0.3	18.4±0.7	174±41	28±3.0

Table 2. Si, Al and Fe content (mean, SD, min and max values, n=10) extractable in ammonium-oxalate and dithionite-citrate-bicarbonate from Giugliano soil samples (X_o, X_d = Si, Al and Fe content extractable in ammonium-oxalate and dithionite, Fe_{est}= estimated ferrihydrite content, Al_o-Al_d/Si_o = Al/Si molar ratio for allophanic materials).

Variable	Mean	Standard deviation	Minimum	Maximum
Si_o (%)	0.01	0.00	0.01	0.02
Al_o (%)	0.34	0.05	0.26	0.44
Fe_o (%)	0.32	0.04	0.25	0.40
Al_d (%)	0.09	0.02	0.05	0.12
Fe_d (%)	0.64	0.21	0.19	0.89
Al_o-Al_d/Si_o	21.2	3.00	17.2	26.9
Al_o+1/2 Fe_o	0.50	0.07	0.39	0.64
Fe_o/Fe_d	0.60	0.12	0.29	1.75
Fe_{est} [b] (%)	0.54	0.07	0.42	0.68

[b] Fe_{est}= 1.7 Fe_o

Table 3. Cu and Zn concentration in extractant solution by different reagents (mean concentration ± SD, n=10) in Giugliano site

	Soil sample	NH_4NO_3 (mg kg^{-1})	EDTA (mg kg^{-1})	Aqua Regia (mg kg^{-1})
Cu	G1-3	8.11±0.2	140±1.5	219±2.3
	G1-5	17.3±0.4	215±5.3	1707±15.3
	G1-6	17.2±0.1	194±5.5	507±12.1
	G1-7	10.2±0.2	171±17.2	614±7.0
	G8-2	1.23±0.0	46.3±1.43	91.3±3.5
	G8-4	16.1±2.4	239±16.1	859±6.0
	G8-5	2.50±0.0	47.6±0.7	96.0±4.2
	G8-7	1.92±0.0	34.1±0.2	108±3.7
	G8-8	1.30±0.8	24.7±0.7	53.5±1.2
	G8-9	0.69±0.5	15.2±0.35	33.4±0.8
Zn	G1-3	1.33±0.0	100±2.8	170±5.2
	G1-5	11.8±0.5	393±14.4	972±6.7
	G1-6	5.24±0.1	332±11.1	591±4.9
	G1-7	1.38±0.0	125±8.6	197±1.7
	G8-2	0.77±0.0	10.8±0.1	80.1±2.6
	G8-4	6.63±0.8	280±27.5	720±6.3
	G8-5	0.61±0.0	36.0±0.8	128±3.0
	G8-7	0.95±0.0	14.4±0.3	84.0±2.1
	G8-8	0.81±0.1	17.8±0.1	79.0±1.6
	G8-9	0.49±0.0	6.48±0.4	58.1±0.9

Table 4. Cu and Zn concentration in extractant solution by different reagents (mean concentration ± SD, n= 9) in Trentola Ducenta site

	Soil sample	NH_4NO_3 (mg kg^{-1})	EDTA (mg kg^{-1})	Aqua Regia (mg kg^{-1})
Cu	TD21-1	4.04±0.2	34.6±1.7	103±4.1
	TD21-5	4.08±0.1	35.9±0.4	63.1±3.2
	TD21-9	4.26±1.6	24.5±1.6	75.1±2.9
	TD32-2	2.71±0.1	17.5±0.3	59.2±2.0
	TD32-4	2.80±0.2	17.5±1.2	51.0±3.7
	TD32-5	3.01±0.3	23.2±0.5	63.2±2.3
	TD32-6	2.02±0.1	12.7±1.3	39.4±1.2
	TD32-7	3.34±0.1	19.5±0.7	42.4±0.9
	TD32-9	1.54±0.0	11.3±0.2	36.0±1.2
Zn	TD21-1	0.57±0.0	46.2±2.4	163±4.0
	TD21-5	0.60±0.0	46.9±0.8	97.7±2.4
	TD21-9	0.58±0.1	39.0±1.9	125±2.6
	TD32-2	0.21±0.0	21.2±0.1	78.5±1.9
	TD32-4	0.54±0.0	40.4±1.8	114±3.1
	TD32-5	0.28±0.0	34.3±1.0	95.5±2.2
	TD32-6	0.09±0.0	18.2±0.7	62.2±1.0
	TD32-7	0.76±0.0	52.8±7.2	228±4.3
	TD32-9	2.58±0.1	139±7.6	256±2.2

Table 5. Leaves accumulation factor (A%) in Eucalyptus camedulensis. leaves and total content in soil of Cu and Zn in Giugliano site (P= plant; CP= compost + plant; CTP=compost + Trichoderma sp + plant)

		A (%)		Total content in soil	
SUB-PLOT	**Treatment**	**Cu**	**Zn**	**Cu (mg kg^{-1})**	**Zn (mg kg^{-1})**
GI1-7	P	1.33	13.8	614±7.0	197±1.7
GI6-5	P	4.82	23.1	106±2.3	84.0±1.2
GI6-6	P	9.96	25.9	109±3.7	79.0±1.0
GI8-3	CP	18.3	41.4	50.0±1.4	71.0±3.2
GI1-5	CTP	1.12	3.49	1707±15.3	972±6.7
GI1-6	CTP	1.71	10.0	507±12.1	591±1.7
GI8-4	CTP	0.78	2.79	859±6.0	720±6.3

Table 6. Correlation coefficients (r) between amounts of Cu and Zn extracted by 1M NH_4NO_3 and 0.05 M EDTA solutions and their total content in soil

	Cu	**Zn**
NH_4NO_3	0.845*	0.979*
EDTA	0.845*	0.967*

* significant with P<0.05

Table7. Correlation coefficients (r) between amounts of Cu and Zn extracted by 1M NH_4NO_3 and 0.05 M EDTA solutions and their content in plants

	Cu	**Zn**
NH_4NO_3	0.391	0.060
EDTA	0.071	0.401

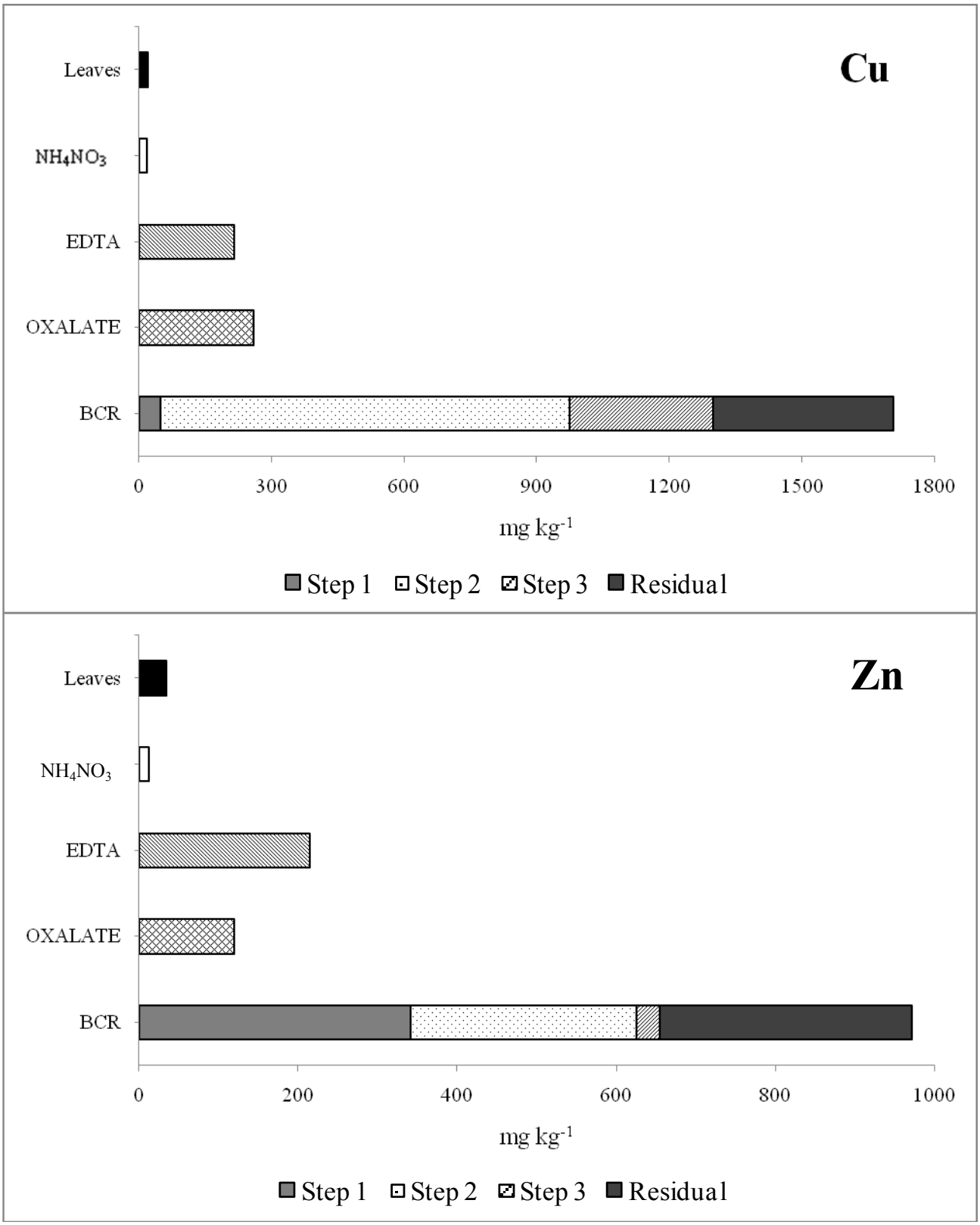

Figure 1. Cu and Zn extracted by single and sequential procedures from G1-5 sub-plot soil in Giugliano site expressed in mg kg^{-1}.(NH_4NO_3= ammonium nitrate; EDTA= ethylenediaminetetraacetic acid; OXALATE= acid ammonium oxalate; BCR= Bureau Comunitarie de Reference-sequential extraction methods from Ure *et al.*, 1993)

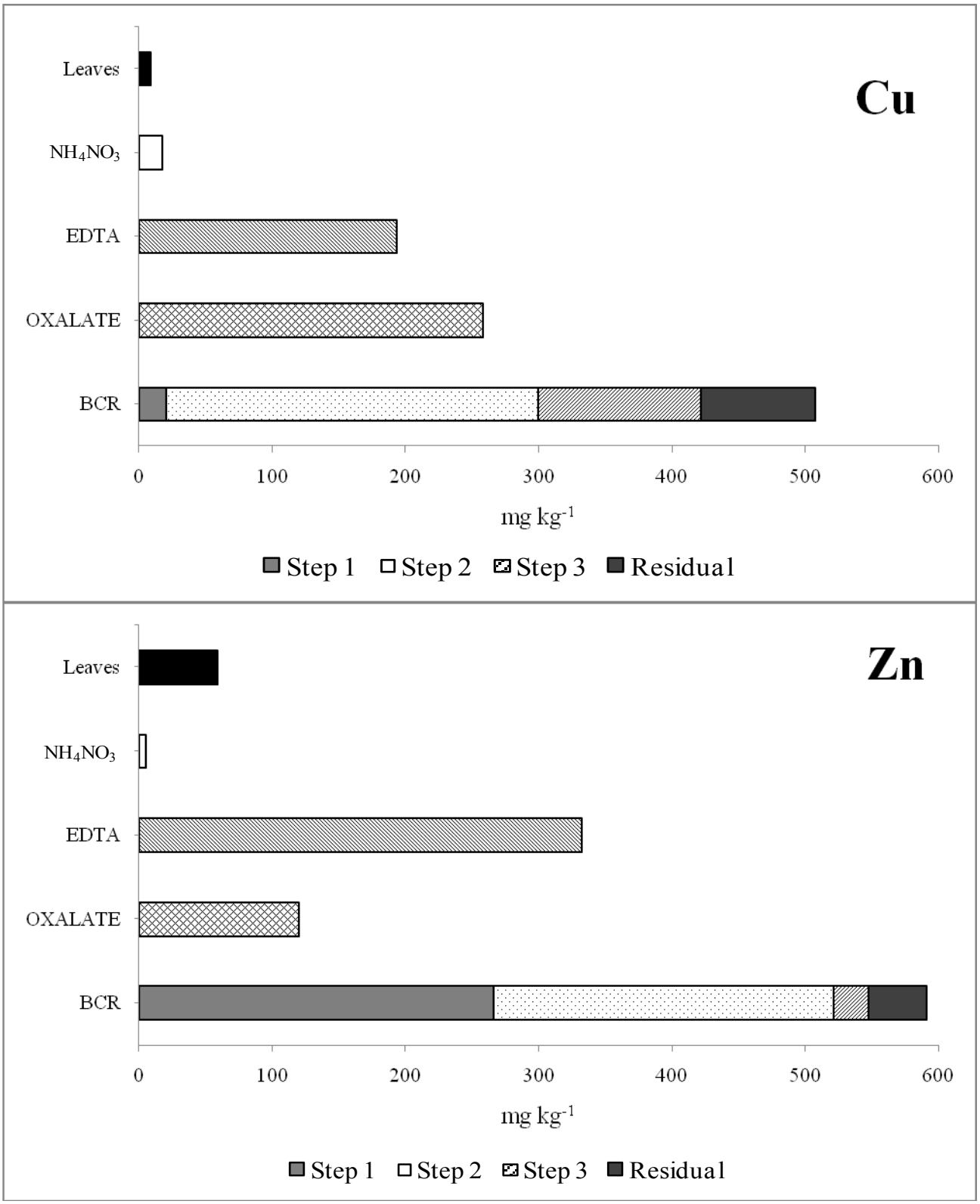

Figure 2. Cu and Zn extracted by single and sequential procedures from G1-6 sub-plot soil in Giugliano site, expressed in mg kg^{-1}.(NH_4NO_3= ammonium nitrate; EDTA= ethylenediaminetetraacetic acid; OXALATE= acid ammonium oxalate; BCR= Bureau Comunitarie de Reference-sequential extraction methods from Ure *et al.*, 1993)

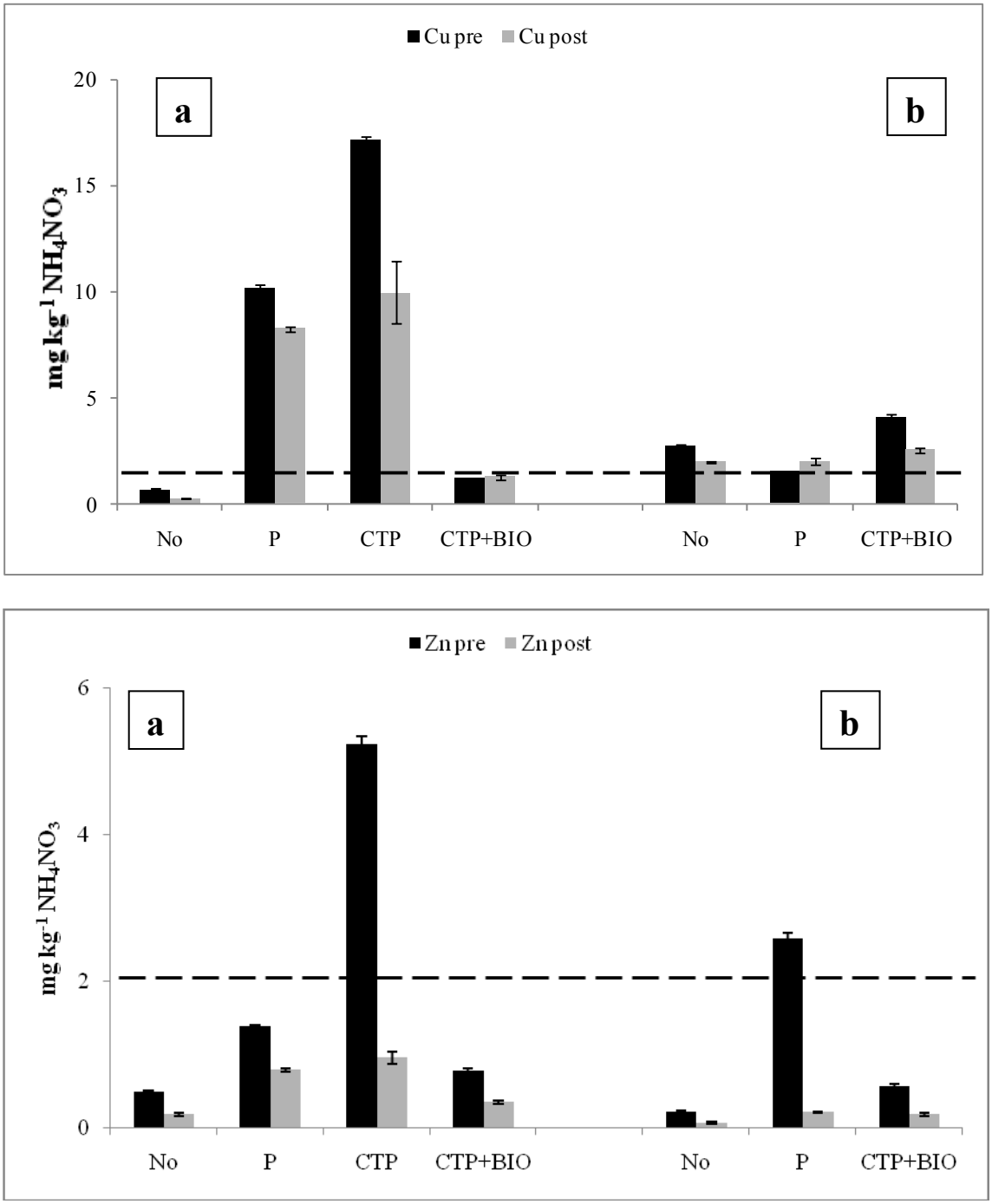

Figure 3. Cu and Zn bioavailable amounts, extracted by 1M NH_4NO_3 before and after one year of phytoremediation treatments in Giugliano (a) and Trentola Ducenta (b) sites. Dotted line = trigger values defined by German DIN 19730 (1997) for the pollutant transition soil-food plant on agricultural areas with regard to growth impairments of cultivated plants (in mg kg^{-1})

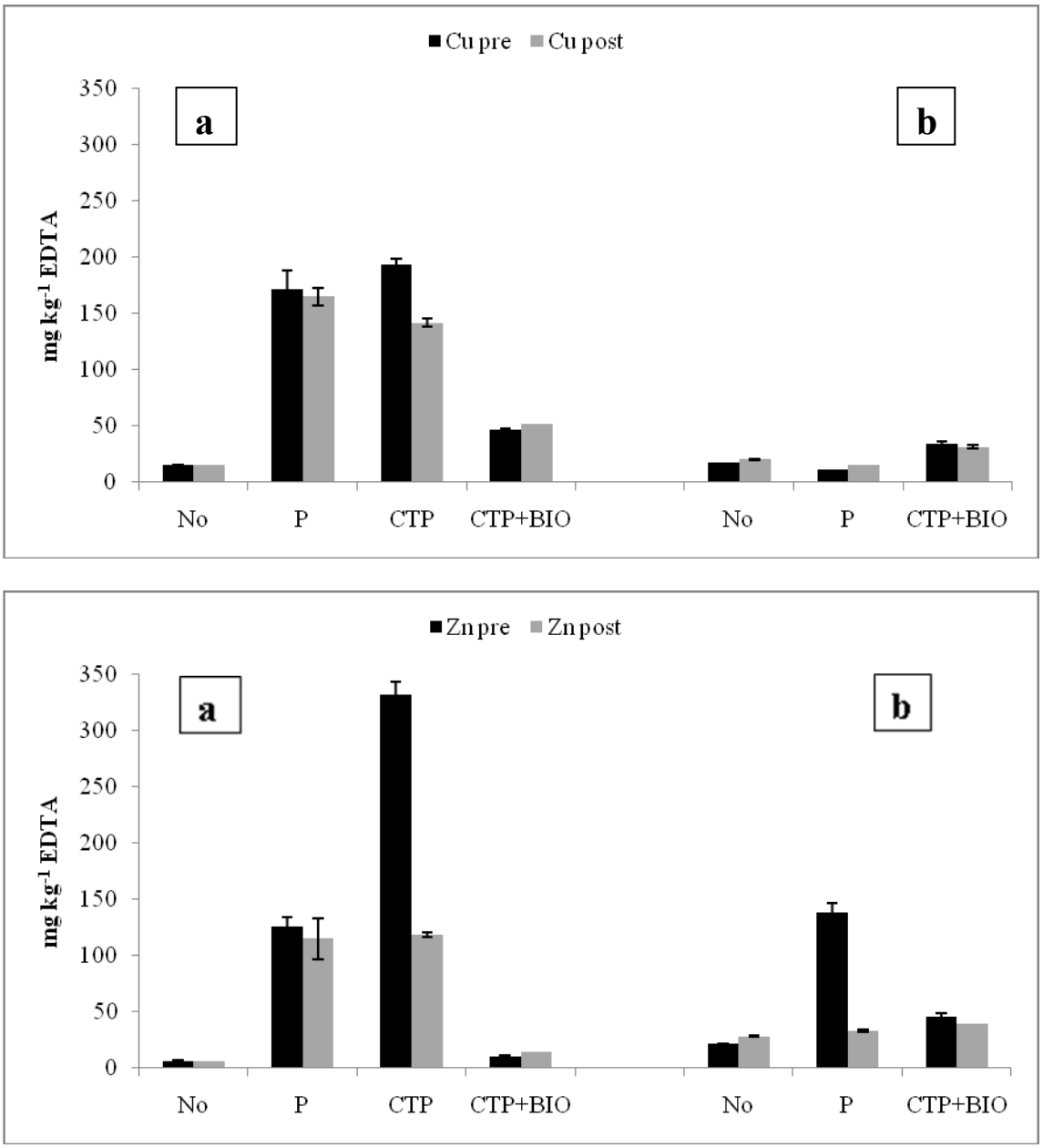

Figure 4. Cu and Zn bioavailable amounts, extracted by 0.05 M EDTA before and after one year of phytoremediation treatments in Giugliano (a) and Trentola Ducenta (b) site.

CHAPTER III

Influence of organic and inorganic amendments on the bioavailability of arsenic and cadmium in soils

1. Introduction

During the past century, the rapid expansion of industrial areas, indiscriminate waste disposal practices, road transport and the use of fertilizers and agrochemicals have led to a relevant increase in global pollution. As a consequence, numerous contaminants are now present in the soils (Janoš *et al.*, 2010). Therefore, heavy metal(loid) pollution is a big problem because these elements cannot be destroyed as some organic contaminants by microbial or chemical degradation, but only relocated. Therefore, their total concentration in soils persists for a long time after their introduction (Bolan *et al.*, 2013). The presence of high levels of toxic and mobile metal(loid)s in the soil, such as arsenic (As) and cadmium (Cd), represents a serious threat for the potential accumulation in the human food chain, essentially by plant uptake and animal transfer (Wuana and Okieimen, 2011). Thus, in the last few decades, there is growing public concern about the potential risks related to the cultivation of As- and/or Cd-polluted soils for agricultural purposes, which lead academia to study and develop cost-effective and eco-friendly technologies able to efficiently remediate polluted areas (Megharaj *et al.*, 2011). Phytoremediation is a solar-driven process for large-scale clean-up of polluted soils by metal(loid) tolerant plants (Caporale *et al.* 2014). For successful phytoremediation, it is crucial to select an appropriate plant species and promote metal(loid) bioavailability, for instance by adding suitable amendments to the soil (Park *et al.*, 2011; Bolan *et al.*, 2013).

However, when phytoremediation may not be applied efficiently (e.g., highly polluted sites), other techniques, such as immobilization (or soil stabilization), should be considered (Park *et al.*, 2011). Kumpiene *et al.* (2007) defined "stabilization" as a remediation technique used to reduce element mobility in soil by the supply of adsorbing agents which reduce leaching to groundwater and plant uptake. In contrast to the classical remediation techniques, immobilization is used to alleviate contaminant exposure with negligible alteration of soil structure and biological activity (Chen *et al.*, 2010).

In agriculture, inorganic and organic amendments are frequently used to improve soil properties. Many of these may significantly modify the mobility of metals (and also nutrients) in the soil environment. Organic waste and animal manures (e.g., poultry manure) have been widely used in the past, as a source of essential nutrients and other benefits to soils and recently, these amendments have been used as mean for improving the remediation of polluted soils (Burton *et al.*, 2003; Park *et*

al., 2011). Thus, the soil amendments, beside their capacity to improve plant nutritional status, can be studied for assessing their influence on the mobility and bioavailability of heavy metals in polluted soils (Janoš *et al.*, 2010).

Bioavailability of heavy metal(loid)s is an important characteristic for better understand their transfer through various systems and the real risk for environmental and human health (Adamo *et al.*, 2008; Bolan *et al.*, 2014).

The aim of this work was to assess the influence of three (i.e., two organic, one inorganic) amendments on the: i) bioavailability of As and Cd in two soils with different physico-chemical properties; and ii) growth and uptake of As and Cd by maize (*Zea mays* L.) plants from two polluted soils.

2. Materials and Methods

In this study, 300 g of clay loam (soil A) and loamy sand soils (soil B) were air-dried, sieved through a 2 mm nylon mesh and separately spiked in plastic bags with 200 mg kg^{-1} of As and 20 mg kg^{-1} of Cd, using $HAsNa_2O_4.7H_2O$ and $Cd(NO_3)_2$ respectively. The levels of As and Cd were chosen on the basis of their cleanup threshold concentration in soils. The spiked soils were incubated for four weeks and mixing thoroughly into the soil to adjust the moisture content to about 70% of water holding capacity (WHC). Thereafter, and for other four weeks, the soil samples were further incubated with organic and inorganic amendments, poultry manure (PM – 10% wt/wt), poultry manure biochar (PMBC – 5% wt/wt), coal fly-ash (CFA – 15% wt/wt). The physico-chemical properties of soil samples and amendments are given in table 1 and 2, respectively.

The incubate samples were analysed for pH using 1:2.5 ratio of sample/distilled water, organic carbon (Walkley and Black method, 1934), cation exchange capacity (CEC) ($BaCl_2$ + TEA at pH 8.1), specific surface area (Quirk *et al.*, 1955) particle size analysis (Andreasen's pipette method) was carried out by wet sieving and by sedimentation in aqueous media after organic matter oxidation by H_2O_2 treatment (according with the official procedures published by Ministero delle Politiche Agricole e Forestali (MiPAAF, 2000)) and bioavailability of As and Cd. Bioavailability was examined using 1M NH_4NO_3 (1:2.5 w/v) and pore-water concentration using rhizon-samplers (Rhizosphere Research Products, Wageningen, Netherlands). On pore water samples was carried out also the dissolved organic carbon (DOC) measurement with TOC analyzer (TOC-L Shimadzu, Total Organic Carbon Analyzer).

In the glasshouse, Stanford and Dement method (1957) was used for examine the effect of soil amendments on the phytoavailability of As and Cd. Maize (*Zea mays* L.) was used as test plant due

to its high ability to extract heavy metal(loid)s in soil (Aliyu and Adamu, 2014) and an high biomass production. In each pot (600 ml capacity; 10 cm diameter) with bottoms removed were nested in similar containers which had intact bottoms and were filled with 500 g sand, 2 seeds of maize were added and Hoagland solution was supplied every day. After 3 weeks, 200 g of spiked soils were placed for five weeks to allow it to equilibrate and to promote adsorption of the added metals. Rhizon-samplers were placed horizontally at 1 cm from the bottom of the pot. Pore-water samples were collected after 2 weeks and analysed for pH and metal(loid)s content. No plant mortality was recorded.

After five weeks the plants were harvested, cleaned with distillate water and put in the oven to 65 °C. The soils were air dried and sieved for remove the roots residue. Plants and soils samples were dried until constant weight. The dry weight of plants were recorded and the plants materials were ground using a stainless steel grinder.

Total content of metal(loid)s were analyzed using acid digestion (HNO_3 for the plants and aqua regia for soils) assisted by microwave oven (EPA 3051 - EPA 3051A) and the extracts were read by emission spectroscopy ICP-OES (EPA 6020A).

All the calculations and standard deviations of the replicates were determined using SPSS version 16.0 (SPSS Chicago, Illinois). Variability in the data was expressed as standard deviation and a $p<0.05$ was considered to be statistically significant.

3. Results and discussions

3.1 Soil characterization and influence of three amendments on the As and Cd bioavailability in the spiked soils before plant growing.

In tables 1 and 2 are shown the pH values, As and Cd content and other properties of the studied soils and amendments, respectively. Basically, two soils presented similar texture, with low background of As and Cd. However, soil A was alkaline (8.5±0.03), maybe for the higher content in carbonates, while soil B was subacid (5.0±0.02) and with a higher organic C content. All amendments contained negligible amounts of Cd and As, except for As in PM (Table 2). Table 3 provides the soil pH after amendments application. Soil treatment with PM and PMBC slightly lowered the pH of soil A (from 8.5 to 7.6-8.0 in the soil spiked with Cd, from 8.5 to 7.8-8.0 in that spiked with As). On the contrary, the pH of soil B increased by addition of all amendments (in particular with PM), with no significant differences between Cd and As spiking.

The Cd content in pore water (Figure 1a) was about 68times increased in soil A after PM application respect to Control and other treatments, probably because of the significant acidification

of soil A (almost a point of pH) by PM treatment, beside the significant release of DOC (Figure 2), which complexing the metal could have enhanced the mobility of Cd in the soil. The mobility and bioavailability of Cd, indeed, are promoted at lower soil pH (Al-Oud *et al.*, 2014; Sherene, 2010). In soil B, Cd content in pore water was strongly lowered with PMBC and CFA application (-0.8 and -0.9 times), maybe due to adsorbing processes. Vice versa, in soil B treated with PM, the higher Cd content in pore water was essentially due to the acidic soil pH and the high release of DOC by PM (Figure 2). In soil A, As content in pore water (Figure 1b) was reduced by PM and CFA application, in comparison to the control and PMBC treatment. In the soil B the presence of As in pore water was quite low; only the application of PM markedly increased As content in pore water which increased 21 times. Differently to the Cd, it is conceivable that the mobility of As is higher in alkaline soil environment (soil A) that in acidic condition (soil B), since As is more strongly bound to variable-charge soil minerals (e.g., Al-, Fe- and Mn-oxides) at lower pH (Caporale and Violante, 2016).

The amount of Cd extracted from soil B by 1M NH_4NO_3 solution (i.e. the easily bioavailable fraction) before plant growing was significantly reduced by all amendants, with slight differences among the treatments (on average -0.9 times) (Figure 3a), probably because the increment of soil pH caused an increase of soil negative charges which may have promoted the adsorption of the toxicant and a simultaneous reduction of its mobility, as also observed by Naidu *et al.*(1994). The abundance of carboxylic and phenolic groups in PM may have contributed to adsorb Cd, further limiting its mobility in the soil B. In soil A, where Cd was probably stabilized by precipitation process (occurring at alkaline pHs), a very low concentration of Cd was extracted by 1M NH_4NO_3 solution; a slight significant increase of Cd bioavailability was produced by PM and CFA application (+4.3 and +1.2 times).

The efficiency of As extraction by 1M NH_4NO_3 was higher in soil A vs. soil B, essentially because of the different mobility of the element in alkaline vs. acidic soil conditions (Figure 3b). Basically, As bioavailability , increased after amendments with PM and PMBC since the release of DOC to the soil made As more mobile (soils A and B), and CFA for possible competition between As and phosphate (released from fly ash) for soil adsorbing sites (soil A).

3.2 Arsenic and Cd content in plant biomass and their bioavailability in soils at plant harvesting time

The amounts of As and Cd taken up and assimilated by maize plants are reported in figure 4. Cadmium content in plants biomass grown in soil A, was slightly lower than in soil B (on average -0.3 times), with no significant differences among different amendments in both soils (Figure 4a).

However, Cd content in plants grown on PM amended soil was slightly lower than those of the other treatments and of Controls, since, as discussed before, PM made Cd less available for plant uptake; this trend was also observed by Bolan *et al.*(2003).

It is noteworthy that maize plants absorbed higher amounts of As than Cd from both soils. Moreover, a significant increase of As uptake was found in plants grown in PM-treated soil B (+0.9 times), probably due to the large presence of DOC that make As more mobile and phytoavailable (Ikhuoria *et al.,* 2010), while a slight (but not significant) reduction of As content was observed in CFA-treated plants (-0.4 times), if compared to the control (Figure 4b). However, Cd and As (only in soil B) amounts taken up by plants was little bit higher than the amounts easily bioavailable, extracted by ammonium nitrate.

After plant growing, the amounts of Cd and As extracted by 1M NH_4NO_3 from both soils followed the same trends observed before plant growing (Figure 5). However, the easily bioavailable fraction of As generally decreased after the growth of maize plants, both in A soil (on average -0.95 times) and in B soil (-0.86 times), because of the significant As plant uptake. Vice versa, for Cd, this reduction only occurred in soil B (on average -0.86 times). It is interesting to note that the lowering in As and Cd easily bioavailable fractions was not perfectly consistent to the amounts of two toxicants taken up by the plants, which may indicate a discreet capacity (buffering power) of both soils to reintegrate the metal(loid) in soil solution. Similar results were also found in pore water, monitoring during plants growing (Figure 6). However, the content of As in pore water of PM-amended soil A in was significantly reduced, probably due to the higher plant uptake.

4. Conclusions

In this study, the influence of organic and inorganic amendments on Cd and As mobility and bioavailability, in two different soils, was assessed. The three amendments (i.e., PM, PMBC and CFA) significantly reduced the mobility and bioavailability of Cd (more available at low pH) in both soils, mainly due to an increase of the pH value that promote the sorption of metals cations and in part to the ability of these material to bind pollutants, reducing their mobility and bioavailability as also obtained in other study (Bolan *et al.*, 2014; Ehsan *et al.*, 2014; Ciccu *et al.*, 2003). Instead, the bioavailability of Cd in soil A was, in part, already mitigated by the higher pH of soil A, independently by the amendments applied. On the contrary, the addition of the three amendments tendentially increased the amounts of As bioavailable, both for the release of DOC from organic compounds and for the release of phosphates from fly ash, which competed with As for soil adsorbing sites. These applications, increased As solubility maybe as results of more high pH and

relatively high carbonate (Brennan *et al.*, 2014; Jackson and Miller, 1999). Arsenic and Cd plant uptake, in general, followed these trends. Only for Cd in soil A and As in soil B, plants adsorbed small quantity above than the amounts "predicted" by ammonium nitrate, confirming the high adaptability and ability of maize to absorb metal(loid)s as also demonstrated by Aliyu and Adamu (2014).

Therefore, the application of organic and inorganic amendments to metal(loid) polluted soils seems to be a feasible and effective way to reduce the mobility and bioavailability of toxicants in the soils, especially for metals quite mobile as Cd. However, the increased availability of As due to the amendments application suggests to deepening the study, taking into account the different nature of the contaminants (cation vs. anion), soil properties and experimental conditions.

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Table 1. Physico-chemical properties and Cd and As content of soils (A=acid and B=alkaline) before spiking and amendments treatments

Soils	pH	CO	Carbonates	CEC	Surface area	Texture (%)			Cd	As
		($g\ kg^{-1}$)	($g\ kg^{-1}$)	($cmol(+)\ kg^{-1}$)	($m^2\ g^{-1}$)	Sand	Silt	Clay	($mg\ kg^{-1}$)	
Soil A	8.5±0.1	15.0±2.8	12.6±4.3	19.7±1.2	156	70	10	20	0.8±0.6	8.5±1.0
Soil B	5.0±0.1	22.0±1.4	3.4±1.3	15.1±0.3	111	75	22	3	0.8±0.7	2.4±2.9

Table 2. pH and Cd and As content in the three used amendments (PM= poultry manure; PMBC= poultry manure biochar; CFA= coal fly ash)

Amendments	pH	Cd (mg kg^{-1})	As (mg kg^{-1})
PM	6.5±0.02	1.0	225
PMBC	7.5±0.04	1.3±0.5	9.1±4.9
CFA	9.9±0.01	BDL*	BDL*

*BDL=Below detection limit

Table 3. pH values of two spiked soils after amendment with poultry manure (PM), poultry manure biochar (PMBC) and coal fly ash (CFA)

Cd	Soil A	Soil B	As	Soil A	Soil B
Control	8.5±0.02	5.0±0.04	**Control**	8.5±0.03	5.1±0.03
PM	7.6±0.05	6.1±0.02	**PM**	7.8±0.10	5.7±0.01
PMBC	8.0±0.03	5.4±0.06	**PMBC**	8.0±0.02	5.5±0.06
CFA	8.4±0.04	5.6±0.06	**CFA**	8.4±0.04	5.7±0.01

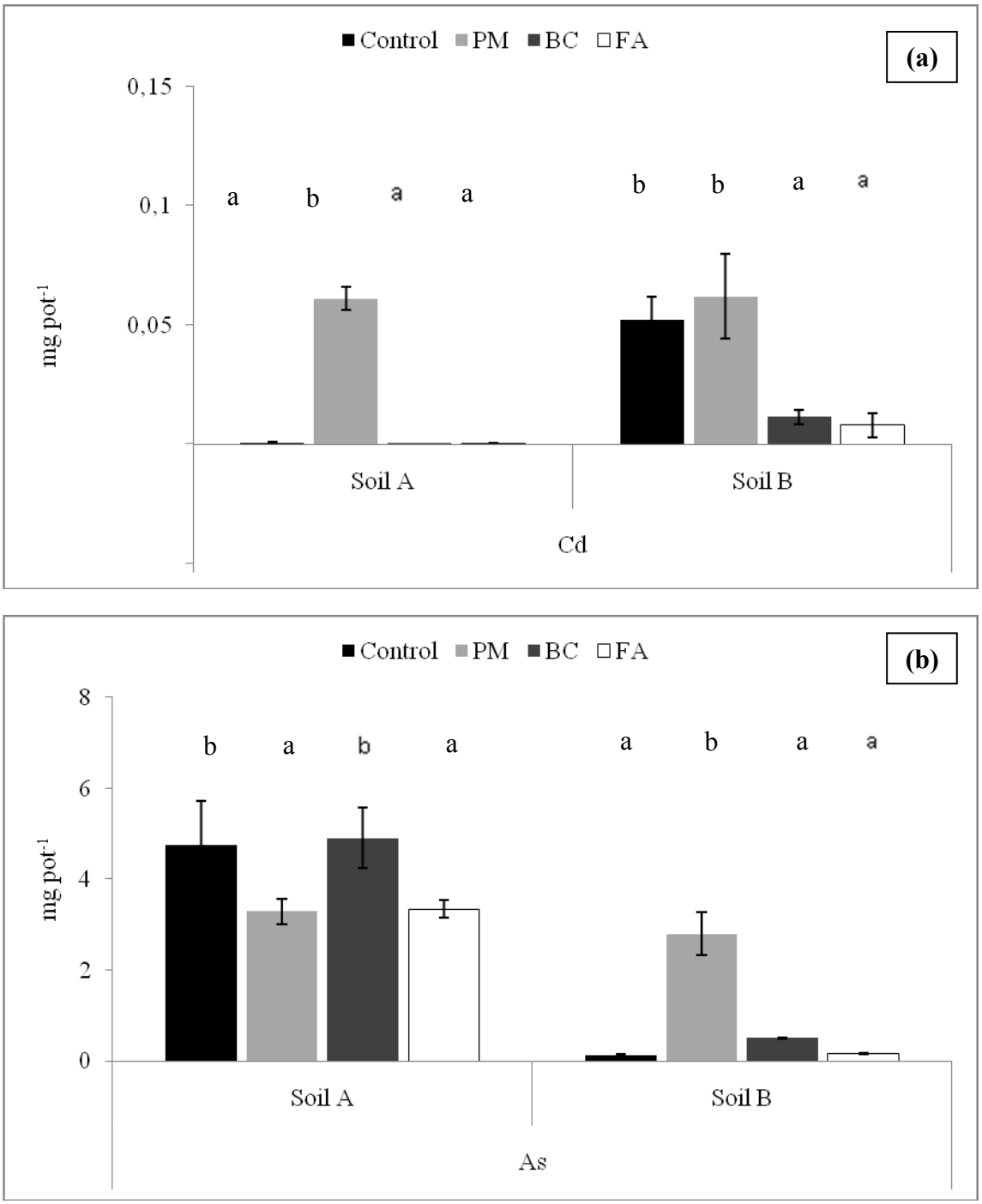


Figure 1. Cadmium (a) and As (b) contents in pore water in alkaline (soil A) and acidic (soil B) soils after amendments application

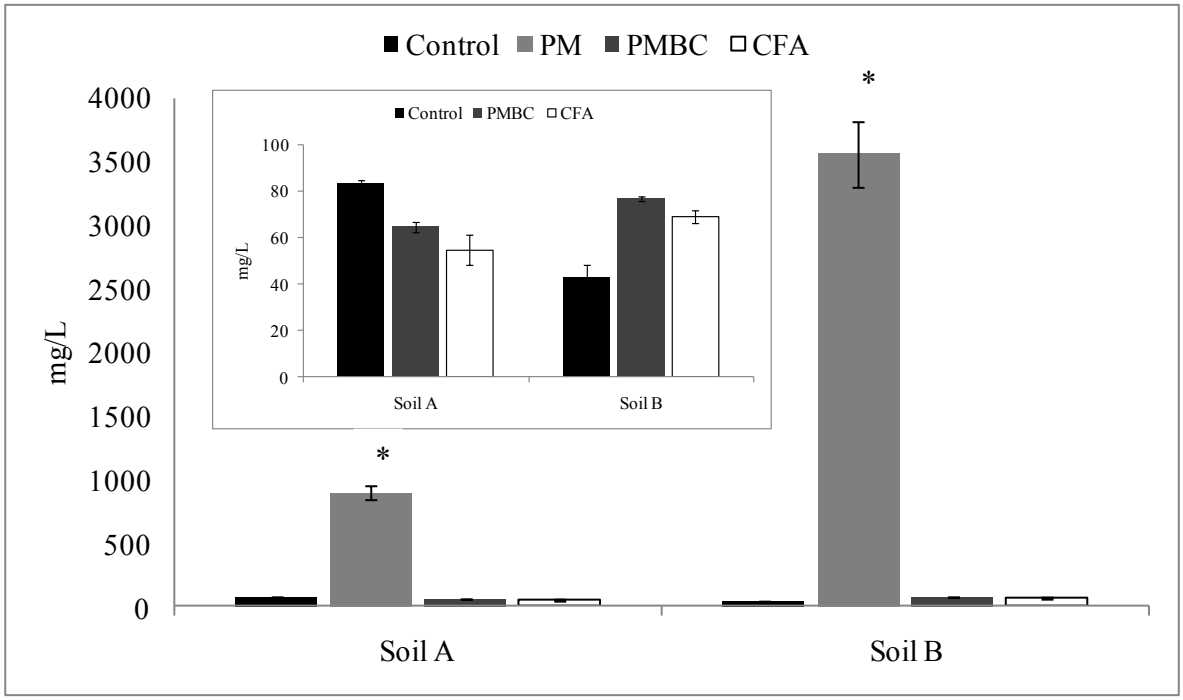


Figure 2. Dissolved organic carbon (DOC) in pore water in alkaline (soil A) and acidic (soil B). In the small box, DOC content in pore water of all treated soils, except PM.

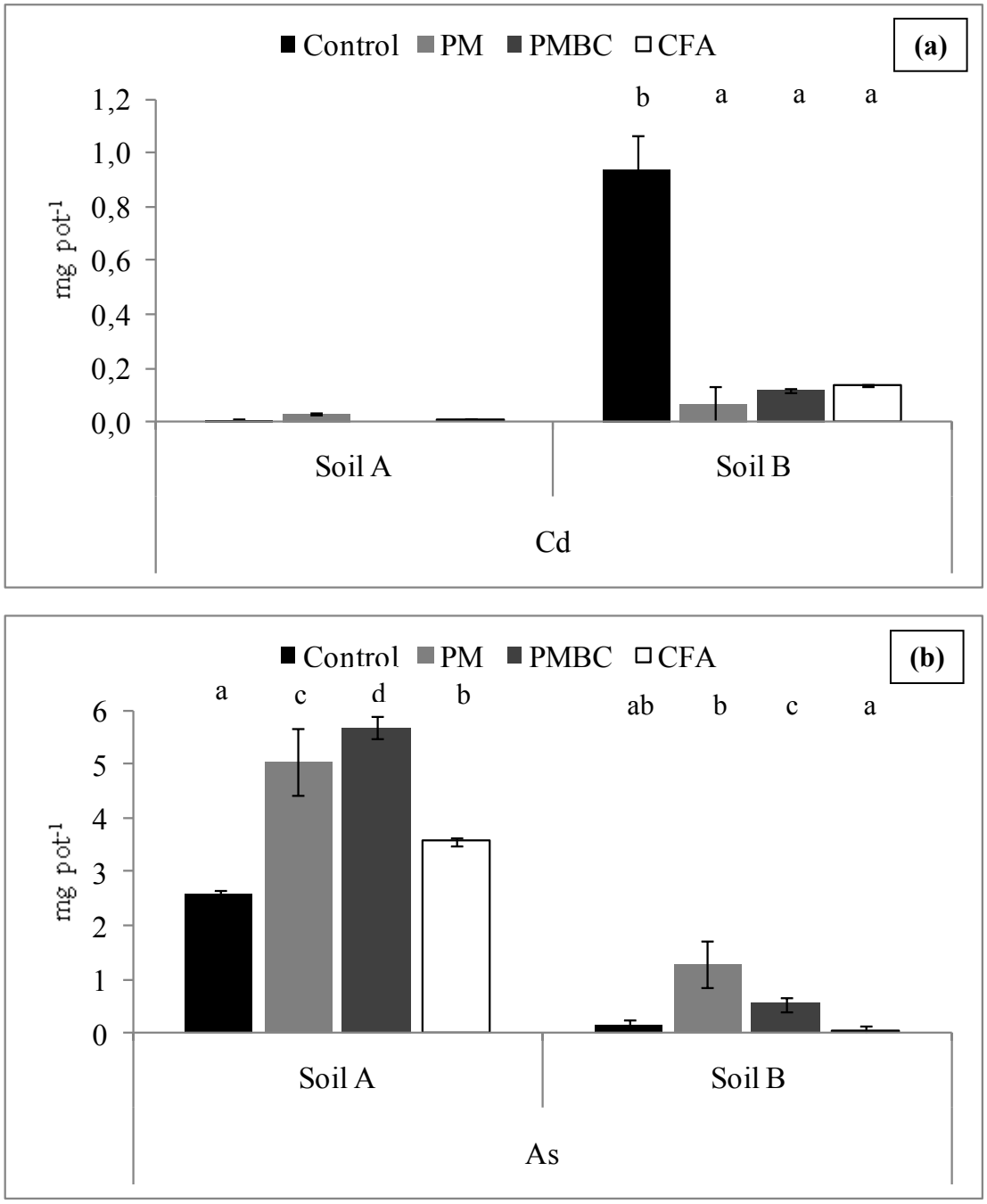


Figure 3. Effect of different amendments on Cd (a) and As (b) (mg pot^{-1}) extracted with 1M NH_4NO_3 before plants growing in alkaline (soil A) and acidic (soil B).

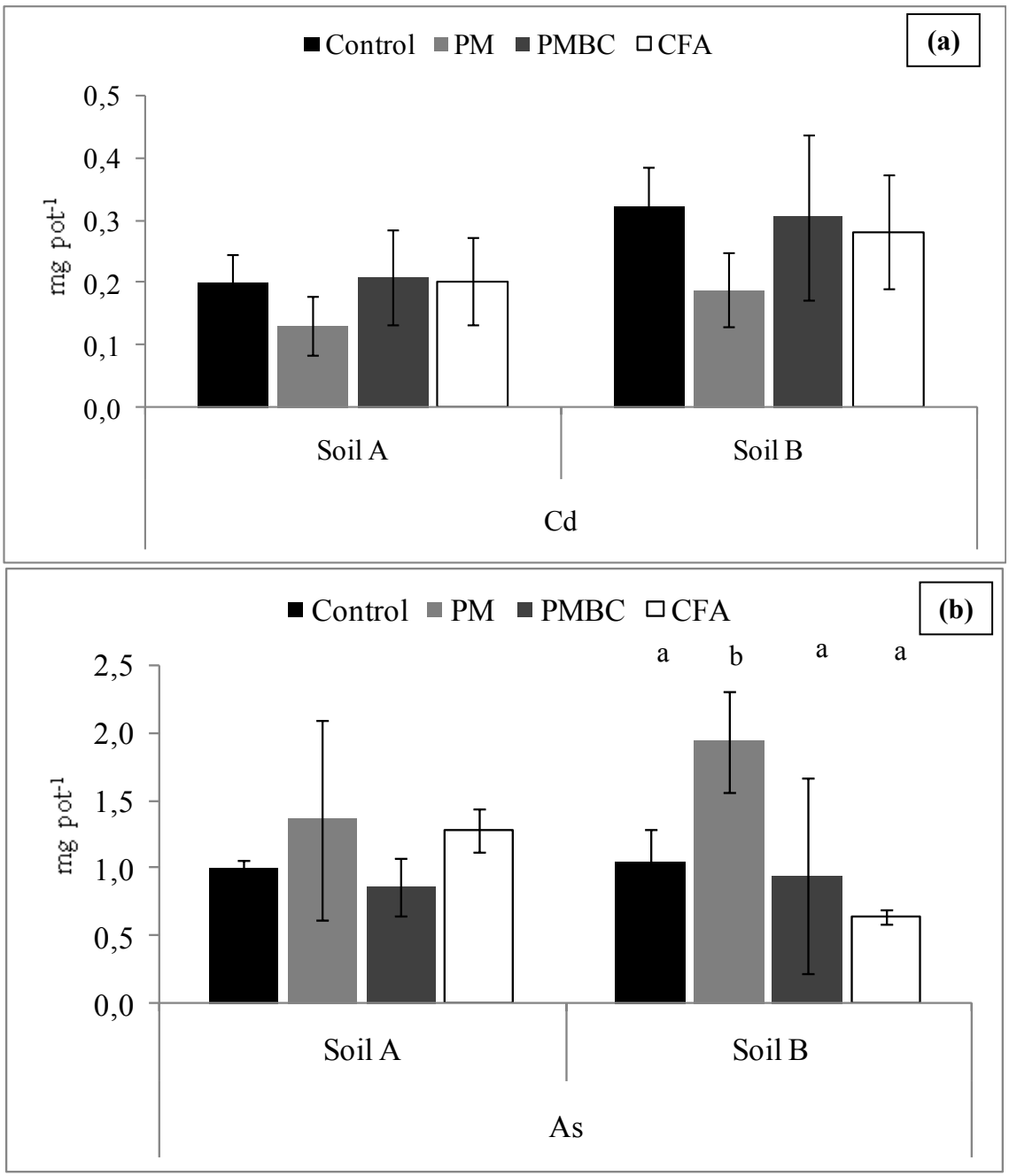
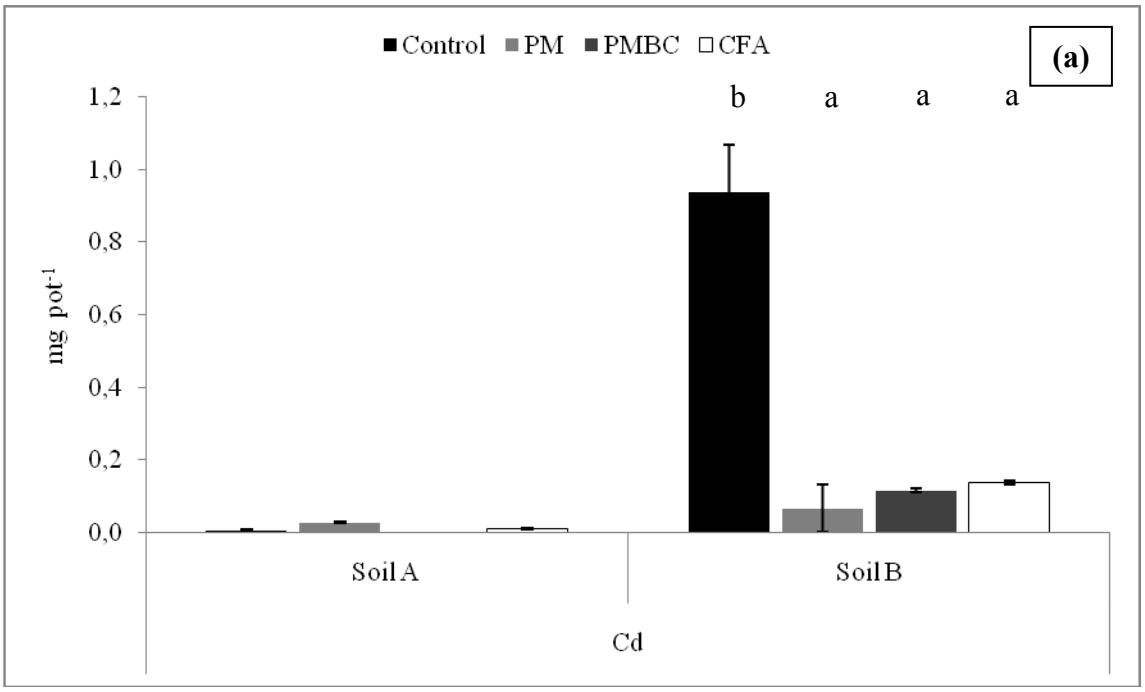


Figure 4. Total content of Cd (a) and As (b) uptaken by plant per pot in alkaline (soil A) and acidic (soil B).



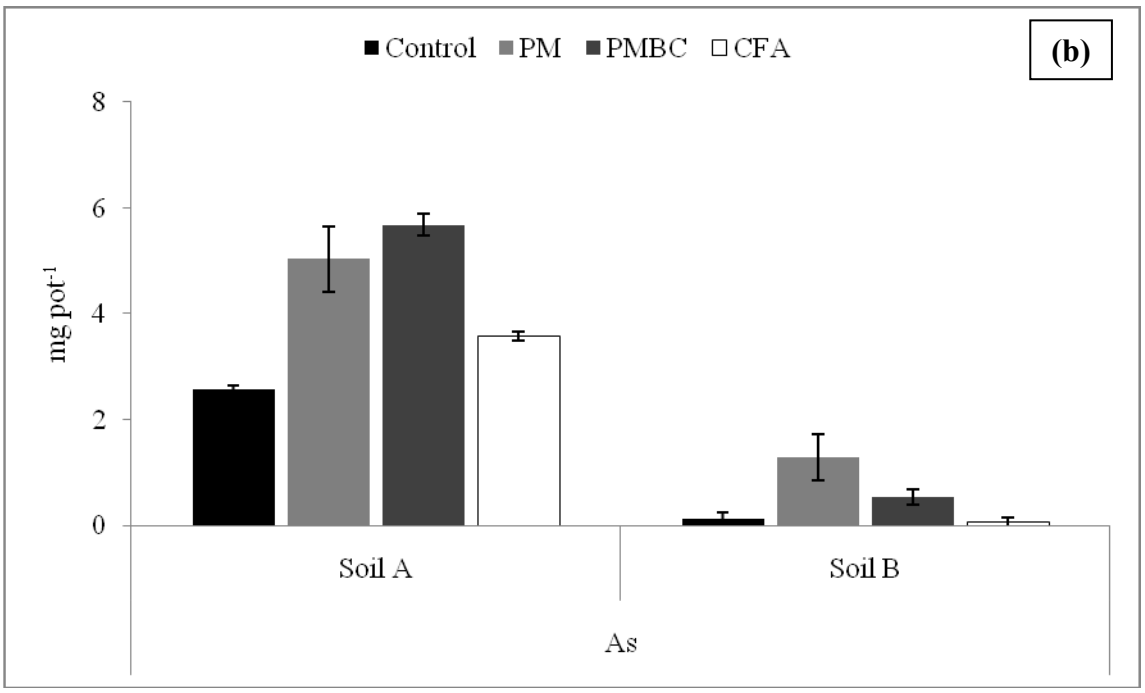


Figure 5. Effect of different amendments on Cd and As extracted with 1M NH_4NO_3 after plants growing in alkaline (soil A) and acidic (soil B).

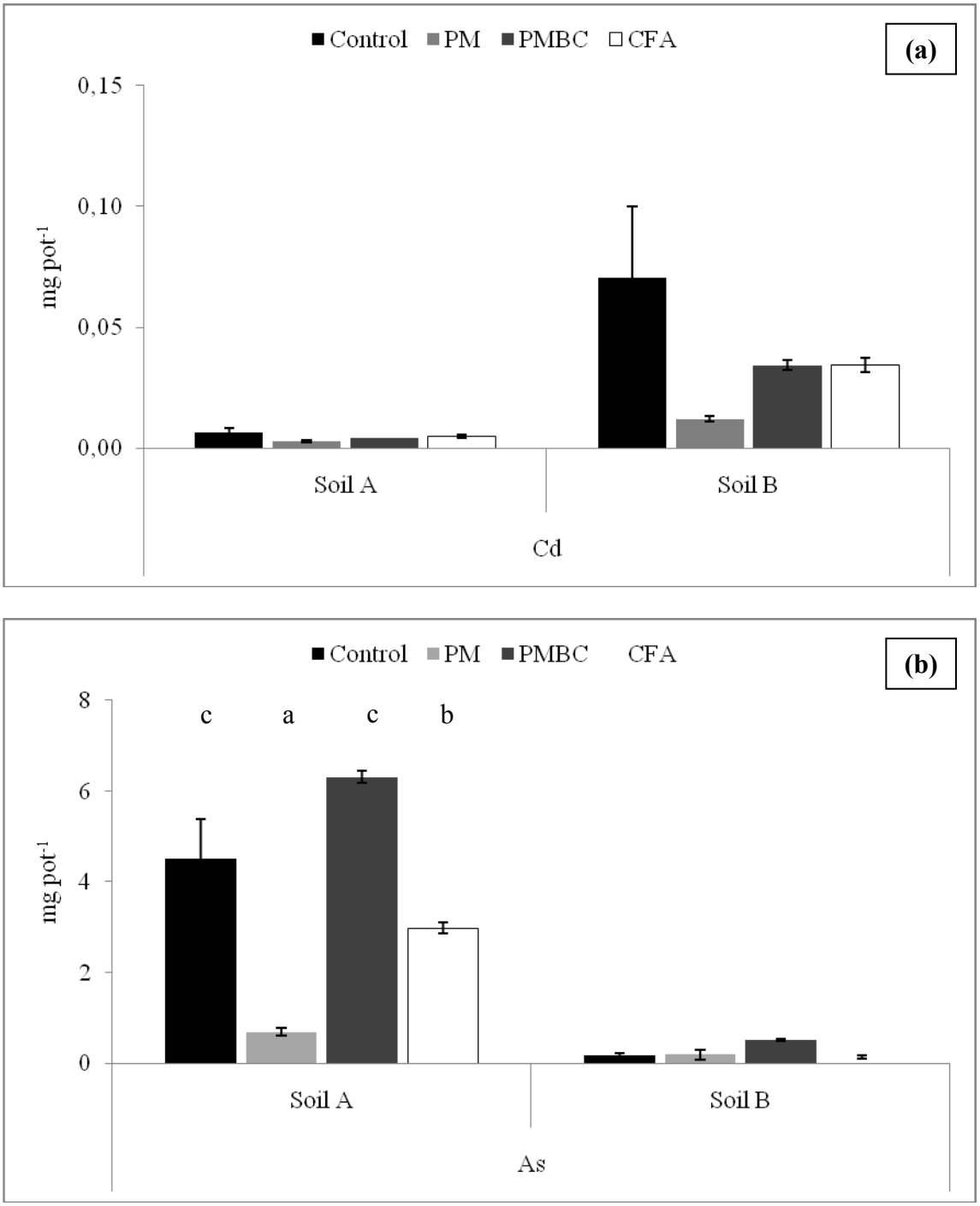


Figure 6. Cadmium (a) and As (b) content in pore water in alkaline (soil A) and acidic (soil B) soils after plant growing

CHAPTER IV

Arundo donax L. adaptability in brownfield soil and sludge: effect of humic acids on plant growth and soil fertility

1. Introduction

During the last decades, the increase of the progress and the industrial activities, with its leakage of industrial waste, have led to serious problems of soil pollution with Potentially Toxic Metals (PTMs) in several part of the world. Due to their high persistence and potential toxicity, the clean-up of polluted soils is very difficult and become a serious problem for human health and environment (Wu *et al.*, 2004). Generally, in residential and agricultural areas, PTMs may be transferred throughout the food chain by intake of vegetation, impacting indirectly on human health through ingestion of both polluted water and foods. Instead, in industrial areas, the risks are mostly linked to air dispersion of polluted soil particles and pollutant leaching toward groundwater. Conventional technologies might be able to remediate polluted soils in relative short time but they are often extremely expensive and may produce a strong negative impact on the environment, causing relevant reduction of soil fertility and biological activity (Lynch *et al.*, 2005; Ensley, 2000).

Soil washing is a common technique used to remedy the brownfield soil and reduce the volumes of soil to be trashed/destroyed. This method uses water (combined or not with solvents) and physical scrub, to remove definitively the smallest particles of soil, which contain the highest quantities of pollutants (García Frutos *et al.*, 2012). Even if soil washing is an easy and cost-effective approach, it produces highly polluted fine products (sludge) suited only to landfill disposal (or for organic pollutants, to thermal destruction) with consequent environmental impacts. For these reasons, alternative treatments are required. In particular, the most cost-effective "green technologies", as assisted phytoremediation technique (amendments and plants application) improve the soil properties, prevent the dispersion of pollutants by water or wind erosion and reduce the PTMs mobility in soil through adsorption and accumulation of moderate level of PTMs by roots or precipitation within the root zone (Adamo *et al.*, 2014; Fiorentino *et al.*, 2013; Fiorentino *et al.*, 2010). Giant reed (*Arundo donax L.*), with its high adaptability to abiotic and biotic stresses, high biomass productivity (from 20 to 40 t ha^{-1}) and stability in a wide range of habitats and nevertheless its high tolerance to pollutants (Fagnano *et al*., 2015; Nassi o Di Nasso, 2013; Angelini *et al.*, 2005), was often chosen as test crop in phytoremediation study. Indeed,

several authors (Fiorentino *et al.*, 2013; Fagnano *et al.*, 2010; Guo and Miao, 2010; Chary *et al.*, 2008; McGarth *et al.*, 2006) had demonstrated that giant reed is an hypertolerant species and, as consequence, it is able to grow well also in highly polluted soil in presence of several PTMs like arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni) and lead (Pb). *A. donax* has also been indicated as one of the most promising crop for bioenergy (Nassi *et al.*, 2011), second generation biofuels and biopolymers (Pirozzi *et al.*, 2015) and cellulose pasta production (William *et al.*, 2010), particularly suitable for the Southern areas of Europe (Fagnano *et al.*, 2015; Lewandowski *et al.*, 2003).

Amendments are frequently used in agriculture to improve the soil properties (Lima *et al.*, 2009). But, also in brown-field sites, characterized by extreme conditions and strong hostility for plant growth, organic amendments could be useful to increase soil fertility. Humic acids (H), are widely used as improvers of plant nutrition and growth, enhancing soil structure and enrichment of soil nutrients increasing the plant-availability of P, Fe, Zn and other nutrients for plant and soil microbial community (Varanini and Pinton, 2001; Valdrighi *et al.*, 1995).

Humic substance may also modify plant metabolism, increasing enzyme activity involved in respiration and glycolysis (Nardi *et al.*, 2007) having a positive effect on photosynthetic metabolism (Vaughan and Malcom, 1983). Several studies (Albuzio *et al.*, 1986; Piccolo *et al.*, 1992; Cannellas and Olivares, 2014) had also demonstrated that humic acid could increase N uptake, stimulate the secondary metabolites production improving plant defence against biotic and abiotic stresses (Schiavon *et al.*, 2010). Humic acid application contributes to an enhancement of organic acids exudation (Puglisi *et al.*, 2008), that play an important role on nutrient availability and uptake and microbial activity in the rhizosphere. Furthermore, the nature of H and its content, play an important role also in the success of remediation and risk attenuation of PTMs-polluted soils (Basta *et al.*, 2005). The PTMs-H interaction is based on the formation of strong metal-humate complexes and influence the reactivity of the metals, including their bioavailability (Ray von Wandruszka, 2000).

This work reports the results of a two-years field-pilot phytoremediation experiment aimed at assessing the adaptability of *Arundo donax* L. to growth on industrial-polluted soils and sludges. Humic acids were tested as improvers of plant performance and soil/sludges fertility.

2. Materials and Methods

2.1 Study site and experimental design

The sampled soils was taken from the brownfield of ILVA (latitude 40°48.570'N, longitude 14°10.557'E, 2-10 m above sea level), situated in the Plain of Bagnoli-Fuorigrotta, part of an active quaternary volcanic system called "Campi Flegrei", in the western urban sector of Naples (southern Italy).From 1910inthis place arose one of the largest Italian industrial areas abandoned by the Italian steel-producing company Italsider in the 1990s and classified as a SNI (Site of National Interest) by the Italian Parliament (2000).

The geological setting of the area is the result of volcanic activity in the Phlegrean Fields, which gave rise to the parent soil material. Within the site, the variability in past industrial activities and material deposition has greatly affected the geopedological status of the area, producing a patchy distribution of soil morphological and pollution features (De Vivo and Lima, 2008; Adamo *et al.,* 2002; Buondonno *et al.,* 1998).

As consequence of an overlap of the past steelworks activities and natural component, potentially toxic metals (PTMs) occur in soil above threshold limit of Italian Ministry of Environment (Italian Parliament, 2006). According to De Vivo and Lima (2008), the upwelling of geothermal fluids enriches in PTM, such As, Cu, Pb and Hg, associated with the Phlegraean fields-active volcanism, provide significant and continuous contribution of metals content present in the soils. A remediation project funded by the Italian government started in 1994 (CIPE, 1994) was mainly based on excavation and soil-washing techniques. For this reason, the soil-washing process is undertaken in a small area of the brownfield site.

A two-years pot experiment from May 2011 to February 2013 was set up in the experimental fields of the Agricultural Department of the University of Napoli Federico II, situated in the Royal Park Gussone of Portici (latitude 40°48.860'N, longitude 14°20.851'E) using the undisturbed soil (S) and sludge obtained after soil-washing (F). The experimental units consist of lysimeters of 0.7 m^3 where giant reed (*Arundo donax* L.) rhizome, collected from the experimental farm of the Department "Torre Lama", were transplanted (May 2011). Rhizomeswere cut to obtain an homogeneous size with 2-3 active buds. After transplant, an half part of both substrates were mixed (or fertilized) with 0.5 g kg^{-1} of a commercial and standardized humic acid (H) from Leonardite (FERTENIA®, Bellizzi, Italy) (O.M 87% d.m.; N 0.75 % d.w.; C/N 78; E.C. 0.16 mS cm^{-1} 18º C; pH 9.4).

Compared treatments resulted from combination of the following factors: substrate (2 levels, S *vs.* F) and fertilization with humic acids (2 levels, H *vs.* NoH). The experimental design was completely randomized with 3 replicates. The lysimeter were freely exposed to the atmospheric agents and during spring and summer of both years the water supply was ensured by weekly manual irrigations ranging from 10 to 15 litres per pot, according to the weather. Any chemical

treatment for the control of phytopathogens was carried out, while weed control was made manually.

2.2 Soil and plant sampling and analysis

Soil (S) and post washing sludge (F) samples were collected at the beginning of the experiment and at the end of each growth season. Bulk-soil was collected 50 cm aside from rhizomes in the 0-30 cm layer while rhizo-soil was collected from roots and rhizome surface. Soil samples were air-dried and sieved at 2 mm prior to the determination of moisture content at 105 °C. pH-H_2O and pH-$CaCl_2$ (1:2.5 soil:water/solution ratio), organic carbon (Walkley and Black method, 1934), nitrogen (Kjeldahl official methods)and carbonates content (pressure Dietrich–Fruehling calcimeter method, Loeppert and Suarez, 1996) were determinate (Table 1and 2).

For testing the bioavailability of PTMs in soil and sludge, two different single extractions were applied. 1M NH_4NO_3 extractant was used to assess the easily available fraction (DIN 19730, 1997), whilst, the PTMs potentially bioavailable fraction was determinate by 0.05 mol/l EDTA solution. PTMs concentration in the solution were determinate by inductively coupled plasma-atomic emission spectrometry Perkin Elmer ICP-AES Optima 7300DV. For this experiment was decided to characterized/analyzed the 15 metals defined by Italian legislation (LD 152/2006).

The above-ground biomass was harvested at the end of each growth cycle while rhizomes were collected at the end of the 2nd cropping cycle. Plant total weight from each experimental unit was splitted in rhizome, culms and leaves. A sub-sample of each tissue was washed with distillate water, weighed and oven-dried at 65 °C until constant weight for dry matter determination and chemical analyses.

Total content of metals and nutrients in rhizomes, culms and leaves were measured using microwave assisted acid digestion in H_2O_2 and HNO_3using a Microwave oven MARS5-CEM. Concentration of all elements in the digestion solution were determinate by inductively coupled plasma-mass spectrometry Agilent ICP-MS 7500ce.Whilst, total content of C and N was determined in CHNS Element Analyzer Fison EA-1108-Thermo Scientific.

2.3 Data analysis

Leaves and culms dry weight (g plant^{-1}) was calculated by multiplying fresh weight by dry matter content. Average culm weight (g culm^{-1}) was calculated by dividing culms dry weight by culm number; culm harvest index was calculated by dividing culms dry weight by aboveground weight, expressing this ratio as a percent.

All data were subjected to an analysis of variance (ANOVA) using a genera linear model (SPSS

version 17.0, Chicago, Illinois). Means were separated according to the LSD sidak test with $p<0.05$.

Data are presented as mean values of three replicates with relative standard errors. All productive data referred to polluted substrates were compared with a not fertilized control grown in the same environmental conditions on an agricultural unpolluted soil.

3. Results and Discussion

3.1 Soil properties

The physico-chemical properties of soil (S) and sludge (F) samples are shown in tables 1 and 2. As expected sludge are mostly made by silt (62%) while soil is sandy (65%). In both substrates the clay fraction never is above 10% (S: 2%; F: 8%). The pH-H_2O was alkaline in both S and F (8.7±0.04; 8.6±0.04, respectively). This is in agreement with their carbonate content (S: 72.6±7.12 g kg^{-1}; F: 60.0±5.78 g kg^{-1}). The soil/sludge pH-$CaCl_2$ was always lower than the pH-H_2O (S:7.9±0.05, F: 7.9±0.01) revealing the reserve acidity of soil colloids.

Organic carbon content was lower in S (9.25±1.26 g kg^{-1}) than in F (15±1.70 g kg^{-1}).

3.2 Crop response and N uptake

F and S showed the same productive level in both cropping cycles with an average aerial biomass of 114 and 241 g DW $plant^{-1}$ in the 1st and in the 2nd cycle, respectively. Aerial biomass grown on polluted substrates (Figure 1) was found lower than control (-16%, on the average of the two cropping cycles), but humic acids allowed a significant increase ($p<0.01$) rising up values (55% and 30% in the 1st and 2nd year respectively) to the same level of not polluted soil.

Culms harvest index (HI, Figure 2a) was found significantly lower ($p<0.01$) in F substrate (72%) with respect to S (84%). Humic acids increased HI to 80% for sludges, while no effect was recorded for S (average value of 84%). A similar pattern was shown by culms weight (Figure 2b) with an average value of 33 g DW $culm^{-1}$ for S regardless of soil amendment, and a significant increase in F treated with humic aicids (from 19 to 31 g DW $culm^{-1}$).

A significant year by soil by fertilization effect ($p=0.04$) was found for N content in culms (Figure 3). At the end of the 1st cropping cycle N content was found at the same level in both fertilized and not fertilized substrates with an average value of 0.32%. An average 0.22 g:100 g increase occurred in the 2nd year for all the compared treatments with the exception of FNoH, whose values were not different from the 1st year.

Biomass accumulation pointed out the plant response to different pollution levels and physical characteristics of the soil. Though Giant reed is well known as hypertolerant to biotic and abiotic stress (Papazoglou *et al.*, 2005), our results clearly show a tendency to lower productive levels on polluted substrates compared to the not polluted control. Our findings are in accordance with those reported by Barbosa *et al.* (2015) showing a Giant reed aboveground production 20% lower (although not different) than unpolluted control, within a pollution range of 450-900 mg kg^{-1} for both Zn and Pb. Nevertheless, in our case biomass reduction was significantly lower than control. Moreover, F substrate resulted more limiting to plant growth than S as proved by a lower harvest index and average weight of culms. Crop stress in F substrate was also highlighted by a constant N uptake (0.40%, on the average) over two consecutive cropping cycles, despite of the higher soil N content (1.1 *vs.* 0.8 g kg^{-1}, for F and S respectively). Most probably soil biological fertility of post washing sludge is limited by the lower oxygen availability and higher metal content of this substrate, thus limiting plant rhizosphere N cycling patterns.

Amendment with humic acids significantly limited plant stress in polluted substrates, increasing biomass production up to levels of not polluted control. Moreover, in F substrate, humic acids positively affected plant HI and average weight of culms, together with N content. This effect was probably due to an improvement of rhizosphere N cycling activity as consequence of an increased root activity and exudation (Smalla *et al*., 2001)

3.3 Metal uptake and partitioning in plant tissues

Lead and Zn content in shoots (Table 3) significantly decreased in the 2nd year, while an opposite pattern was shown by aboveground removal (mg $plant^{-1}$) with an average increase of 80% and 55% for Pb and Zn, respectively. This behavior was mainly related to the increased plant biomass produced during the 2nd year. F substrate showed higher metal content and uptake in aboveground tissues, with a marked difference especially for Zn, correlated to the higher initial total content of Zn in F respect to S. No significant effect was recorded for fertilization, with the exception of Zn removal of culms that increased from 6.5 to 9.4 mg pt^{-1} with the addition of humic acids. Lead values were found one order of magnitude higher than control in leaves (0.39 vs. 0.034 mg kg^{-1}) while culms were at the same level (0.68 vs. 0.59 mg kg^{-1}). Zn content was found 3 times higher than control in culms (49 vs. 9 mg kg^{-1}) and 5 times higher in leaves (77 vs. 16 mg kg^{-1}).

Year by substrate interaction for Zn content of aboveground tissues is shown in figure 4a. Tissues grown on S did not show any variation over years with an average value of 39 and 59 mg $kg^{-1}$of Zn in culms and leaves, respectively. On the contrary, Zn values recorded for F significantly decreased in the 2nd cropping cycle in both culms (from 105 to 60 mg kg^{-1}) and leaves (from 127

to 90 mg kg^{-1}). Average Pb and Zn content in rhizomes is shown in figure 4b. Recorded values were significantly higher in F substrate, nevertheless Zn values were significantly higher than control both in F (121 vs. 25 mg kg^{-1}) and in S (55 vs. 25 mg kg^{-1}). On the contrary, Pb content was found at the same level of control in S (5 mg kg^{-1}) and 2.5 times higher in F (13 vs.5 mg kg^{-1}). According to these results rhizomes and then leaves are the main sink for Zn in Giant reed, while Pb was mainly stored in rhizome. This pattern can be ascribed to the different metabolic relevance of the two metals. Zinc plays a key role in enzymatic activities as dehydrogenases, proteinases, peptidases and phosphohydrolases and high levels of this metal are reported in aboveground biomass of several crops grown in polluted sites (Kabata and Pendias, 2001). On the contrary Pb, does not play any significant role in plant metabolism and is mainly accumulated in root vacuoles with a translocation to shoot lower than 3%. Moreover, high soil pH values as those of our experiment may precipitate Pb as hydroxides, phosphates, or carbonates, as well as promote the formation of Pb-organic complexes that are rather limiting metal availability to plants (Kabata and Pendias, 2001). In addition our results highlighted a preferential allocation of Zn in Giant reed rhizomes at a higher soil Zn content. Despite of a aboveground biomass increase of +111% in the 2nd year, no reduction in Zn content of aboveground tissues (40 mg kg^{-1}, on the average) occurred in S (315 mg Zn kg^{-1}), while a significant decline (from 100 to 60 mg Zn kg^{-1}) was found for F. Since an equal increase in aboveground biomass production occurred for both substrates in the 2nd year, the dilution of aboveground Zn content in F presumably occurred because Zn availability to plant in F substrate overcomes plant requirements with a consequent limitation of the metal toxicity through metal compartmentalization in roots (Kabata and Pendias, 2001).

3.4 Metal bioavailability

Average bioavailable Pb and Zn recorded at the end of the 2nd cropping cycle compared to the beginning of the experiment are presented in figure 5. Bioavailable amounts of Pb and Zn were significantly higher in F than in S with the exception of NH_4NO_3 extracted Pb (data not shown) whose values were found at the same level for both F and S (0.14 mg kg^{-1}, on the average). EDTA extracted Zn was found 7 times higher in F substrate (144 vs. 21 mg kg^{-1} for F and S, respectively), due to the different total content of Zn in the two substrates, and no interaction was found between substrate and humic acids.

On average the EDTA extracted fraction from F substrate decreased at the end of the 2ndcropping cycle with a variation of -26.4 mg kg^{-1} and -46.4 mg kg^{-1} for Pb and Zn, respectively. No significant variations were recorded for S substrate.

The reduction of the EDTA extractable fraction of both metals in F at the end of the experiment suggests a change in the distribution of Pb and Zn among the soil geochemical fractions. Probably the higher organic matter content of sludge and the reduced root-plant interaction in this substrate might have favored the immobilization of the metals during the two years experiment.

Soil organic carbon content of bulk soil (Figure 6) did not show significant variation at the end of the 2nd cropping cycle, and no effect was detected for fertilization with humic acids. SOC content in the rhizosphere was significantly higher only for S substrate (+2.6 g kg^{-1}), while no difference was recorded in F substrate.

SOC content in bulk and rhizo-soils, reveals how the low biological fertility of F soil limits the common root-soil interactions. It is well known that root exudation is a consistent source of easy available carbon which increases biotic activity of rhizosphere (Sørensen, 1997). In our experiment this effect was recorded only for S, while no increase of rhizosphere SOC content was found in F suggesting a reduced root-plant interaction in these substrate. This result is in accordance with the low N availability in post washing sludge not treated with humic acids.

4. Conclusions

Giant reed (*A: donax*), is well known as hypertolerant plant and widely used in other phytoremediation studies because of its high adaptability to grown on polluted soils and high capacity to hyperaccumulate pollutants in its tissues.

In this study the application of humic acid to PTMs industrial polluted soil and sludge was shown to enhanced the adaptability and grown of Giant reed.

A lower plant productive levels were observed on polluted substrate respect to those related to the unpolluted soil, and F substrate was more limiting to plant growth than S. The amendments application significantly increased the biomass production, in both soil and sludge. In particular, although *A. donax* grown on more polluted substrate, i.e. sludge, it was interesting to observe an increasing of plant biomass as a consequence of humic acid addition. Indeed, the presence of humic acids positively influenced plant harvest index, average culms weight and N content, maybe due to an improvement of rhizosphere N cycling activity consequent to more intense root activity and exudation.

The addition of humic acid, although useful for the plant growth, had no influence on Pb and Zn mobility and bioavailability, regardless of substrate type. Other factors may influenced the PTMs mobility in soil, especially the pH, whose high values could reduce the metals bioavailability (e.g., for metal precipitation). Despite of this result, the amounts of metal uptaken by *A. donax* was

above than those “predicted” by ammonium nitrate extraction, confirming its high ability to phytoextract metals in all conditions and, hence, encouraging its use for phytoremediation purposes.

In the light of all these results, it is possible to consider dismissed industrial lands potentially useful for bioenergy production by cultivation of plant with high biomass, likely *A. donax*.

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Table 1. Main physico-chemical properties of soil (S) and post-washing sludge (F) before treatments

	Soil Texture (%)			pH	Carbonates	N Org	C Org	C:N	Pb	Zn
	Sand	Silt	Clay		(%)	g kg^{-1}	g kg^{-1}		mg kg^{-1}	
S	65	33	2	8.7±0.04	6.5	0.80	8.90	11.1	168	315
F	30	62	8	8.6±0.04	5.7	1.10	12.2	11.1	270	1139

Table 2. Main physico-chemical properties of rhizo- and bulk- soil (S) and post-washing sludge (F) after plants and humic acid (H) application.

		pH-H_2O	**pH-$CaCl_2$**	**Carbonates (g kg^{-1})**	**OC (g kg^{-1})**
Rhizo	SNoH	8.7±0.05	7.9±0.03	80.6±6.46	13.2±3.00
	SH	8.7±0.02	7.9±0.01	75.2±4.19	12.0±0.86
	FNoH	8.7±0.10	7.9±0.03	70.9±6.12	14.6±2.99
	FH	8.7±0.10	8.0±0.02	62.5±8.43	17.3±1.56
Bulk	SNoH	8.7±0.04	7.9±0.05	72.6±7.12	9.25±1.26
	SH	8.7±0.02	7.9±0.01	79.6±5.78	8.79±0.87
	FNoH	8.6±0.04	7.9±0.01	60.0±5.78	15.8±1.70
	FH	8.6±0.13	8.0±0.04	63.7±5.31	15.2±1.52

Table 3. Comparison of Pb and Zn content in culms, leaves and shoot of *Arundo donax* L., in two years (1st and 2nd cycle), two substrates (F and S), in presence or absence of humic acid (H and NoH)

	Pb (mg kg^{-1})			Zn (mg kg^{-1})			Pb (mg pt^{-1})			Zn (mg pt^{-1})		
	Culms	**Leaves**	**Shoot**	**Culms**	**Leaves**	**Shoot**	**Culms**	**Leaves**	**Shoot**	**Culms**	**Leaves**	**Shoot**
1st year	0.42	0.68	0.48	71.4	92.5	76.6	0.035	0.019	0.054	5.80	2.60	8.40
2nd year	0.36	0.67	0.41	50.1	74.9	54.2	0.072	0.025	0.097	10.2	2.80	13.0
F	0.46	0.71	0.52	82.5	109	88.6	0.060	0.026	0.086	9.80	3.80	13.7
S	0.32	0.65	0.38	39.0	58.6	42.2	0.047	0.018	0.065	6.10	1.60	7.80
H	0.35	0.65	0.41	60.8	79.6	64.5	0.060	0.023	0.083	9.40	2.70	12.2
NoH	0.43	0.71	0.49	60.7	87.8	66.3	0.047	0.021	0.068	6.50	2.70	9.30
Y	*n.s.*	*n.s.*	*	**	*n.s.*	**	**	**	**	**	*n.s.*	**
S	*	*n.s.*	*	**	**	**	*n.s.*	**	*n.s.*	*	**	**
F	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*	*n.s.*	*n.s.*

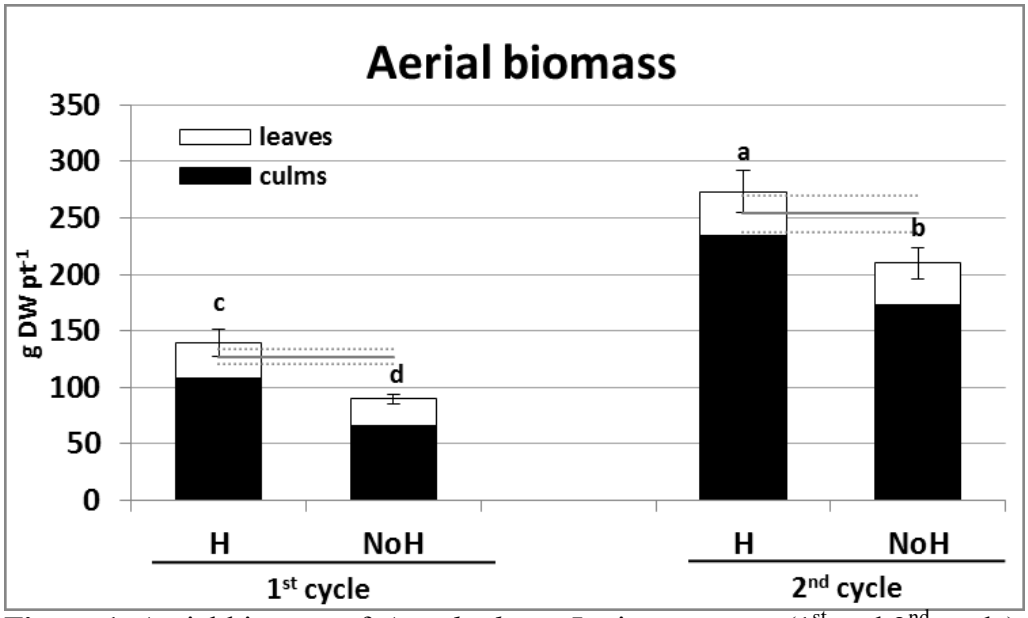

Figure 1. Aerial biomass of *Arundo donax* L., in two years (1st and 2nd cycle), grown in presence (H) or absence (NoH) of humic acids. Horizontals bars indicate the aerial biomass of plants grown on unpolluted soil.

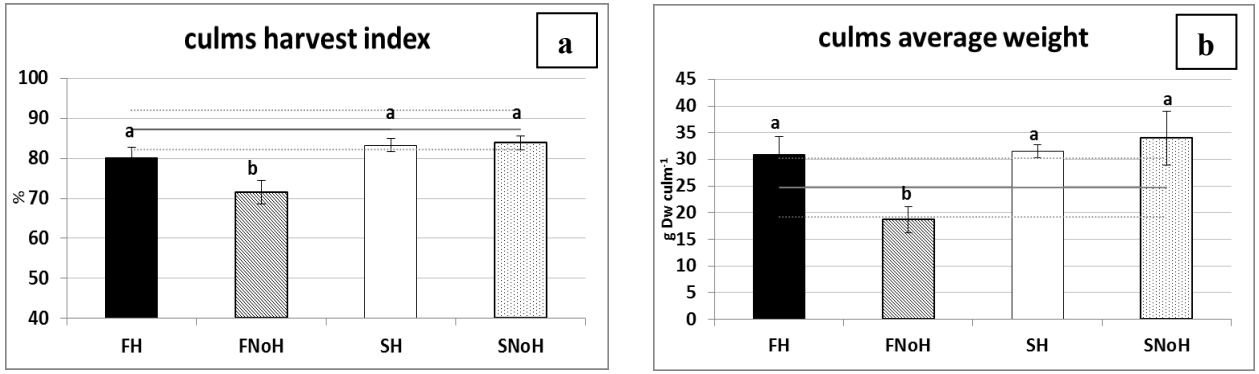

Figure 2. Culms harvest index(a) and culms dry weight (b) of *A. donax* L., in post-washing sludge (F) and soil (S), grown in presence (H) or absence (NoH) of humic acids. Horizontals bars indicate the aerial biomass of plants grown on unpolluted soil.

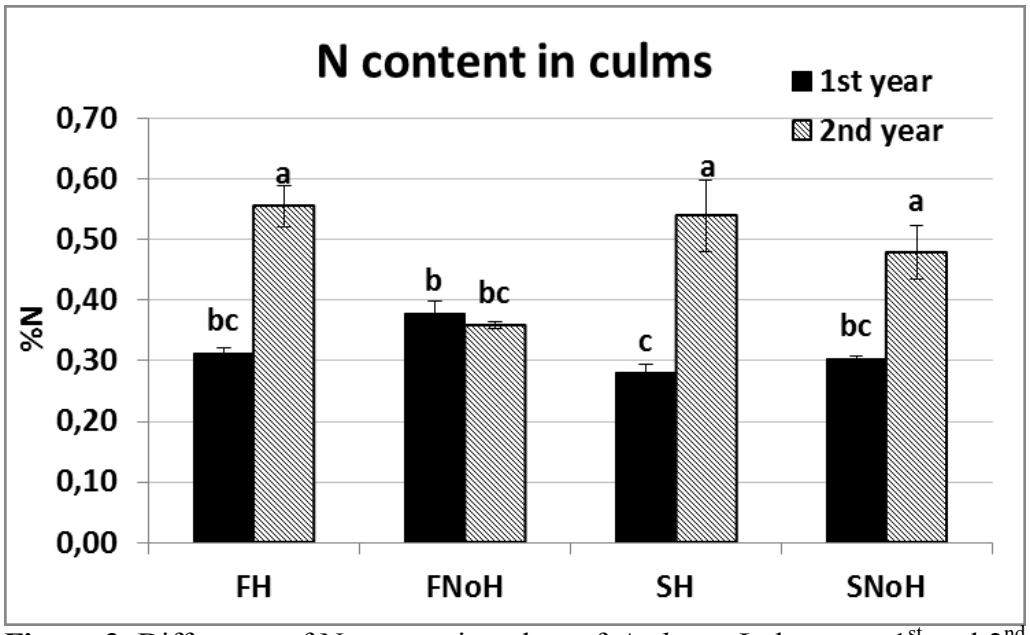

Figure 3. Difference of N content in culms of *A. donax* L. between 1st and 2nd year, in post washing sludge (F) andsoil (S), grown in presence (H) or absence (NoH) of humic acids.

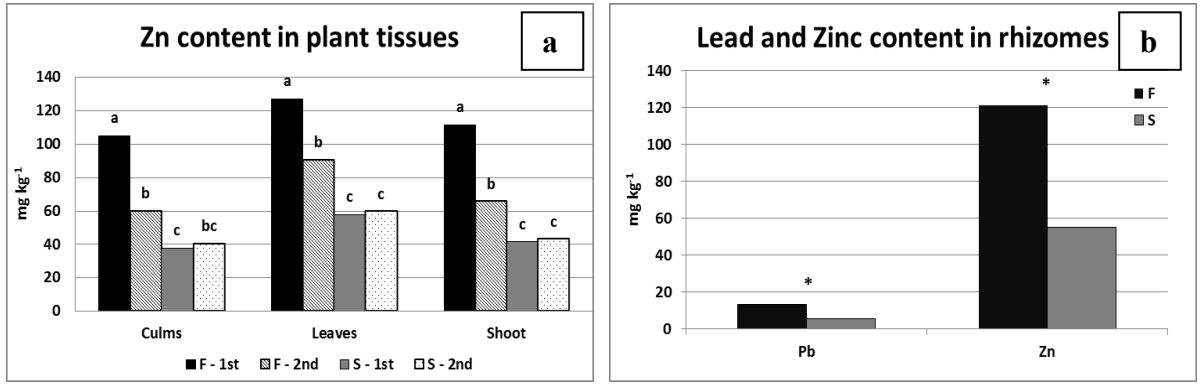

Figure 4. Year by substrate (S and F) interaction for Zn content (a) in aboveground tissues and average of Pb and Zn contents in rhizomes (b) of *A. donax* L.

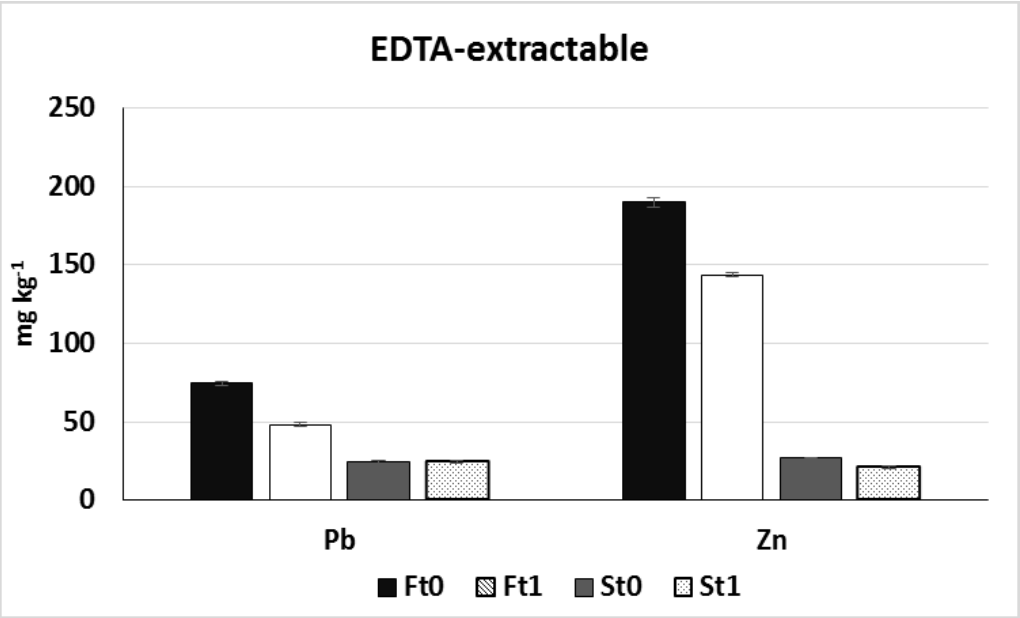

Figure 5. Bioavailable amount of Pb and Zn, extracted by EDTA solution, at the beginning of the experiment and after 2ndcropping cycle in post washing sludge F and soil S.

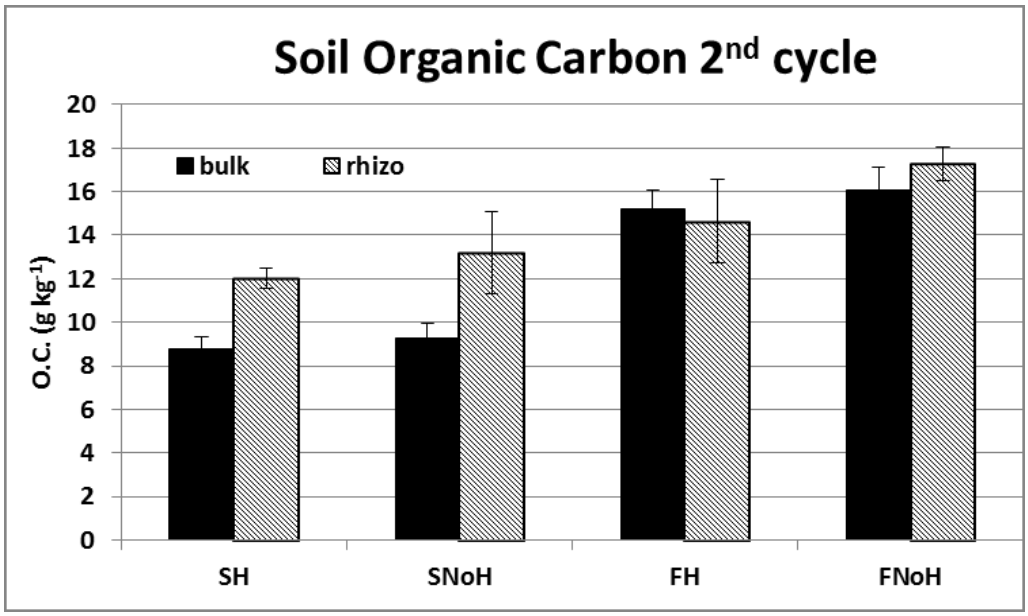

Figure 6. Comparison of soil organic carbon content between bulk- and rhizo-soils/sludges at the end of 2nd cropping cycle.

GENERAL CONCLUSIONS

The choice of an appropriate sampling scheme is a crucial step in the process of soil pollution assessment and risk management. In the first study presented in this thesis, a 3x3 m high resolution grid (discrete sample) and a regular 10x10 m low-resolution grid (obtained by bulking of 3x3 m discrete samples) were applied in three agricultural sites suspected of heavy metal pollution by waste disposal. The aims were to investigate the spatial distribution patterns of pollutants in soil, to highlight the difficulties to assess appropriately the degree of soil pollution and the detect pollution foci when large scale sampling is applied. From the obtained results, only one site appeared to be polluted, mainly to Cu and Zn. A large inhomogeneities in soil pollutant spatial distribution emerged from comparison of data taken at high and low resolution level. Also unpolluted 10x10 m plots were found to contain metals above legal limits when sampled at 3x3 m scale. The degree of soil pollution, as assessed by pollution indices, worsen passing from 10x10 m to 3x3 m scale of sampling. According to our results, when an high spatial variability is suspected, the risk to underestimate the pollution degree and omit pollution foci is elevated. By contrast, a high resolution soil sampling is very expensive and arduous when the monitoring of soil contamination has to be done over large areas. An appraisal of the site history aimed to identify past and present potential contaminating activities and preliminary geophysical measurements might help to define the most appropriate sampling scheme.

In order to assess the Cu and Zn mobility/bioavailability and to predict the feasibility and the effectiveness of phytoremediation treatments in the above studied agricultural polluted soils, a combination of single and sequential extraction procedures were applied. The readily and potential mobility and bioavailability of both metals, higher for Zn than for Cu, was a reliable indication of the suitability of plant cropping as soil remediation treatment. Indeed, after one year from *Eucalyptus camedulensis* planting, a significant reduction of the bioavailable amounts of Cu and Zn in soil, as assessed by single chemical extractions, was observed in both sites without any significant difference between the applied treatments. From the comparison between the Cu and Zn amounts extracted by 1M NH_4NO_3 solution and those uptaken by plants (leaves content), a general underestimation of bioavailable Zn as assessed by chemical extractions was observed. A more detailed information come from the sequential extractions, indicating that Zn was mainly associated to the easily HOAc-extractable and reducible fractions, while Cu was mostly found in reducible and oxidizable phases. Thus, for an appropriate characterization of polluted soils we would suggest to combine single and sequential chemical extractions in order to obtain a more accurate description of the chemical status of metals in soil and therefore a

better prediction of their bioavailability. In our case the removal of metals from the soil by *Eucalyptus* is an evidence that, even if in a consistent number of years, the chosen phytoremediation treatment should clean up the soil reintegrating it to the original agricultural use devoted to food production.

The addition of the organic and inorganic amendments (PM= Poultry Manure, PMBC= Poultry Manure Biochar and CFA= Coal Fly Ash) to two different Australian soils significantly reduced the mobility and bioavailability of Cd (occurring in soil predominantly as a divalent cationand more soluble at low pH) in both soils, mainly due to an increase of the pH value that promote the sorption of the metal and in part to the ability of these materials to bind the pollutant, reducing its mobility and bioavailability. On the contrary, the addition of the three amendments basically increased the amounts of bioavailable As (occurring in soil predominantly as trivalent and pentavalent anions**)**, both for the release of DOC from organic compounds and of phosphates from fly ash, both competing with As for soil adsorbing sites. These applications increased As solubility likely as a result of the higher pH and relatively high carbonate content. Arsenic and Cd plant uptake, in general, followed these trends. Therefore, the application of organic and inorganic amendments to Cd and As polluted Australian soils characterized by different physico-chemical properties, seems to be a feasible and effective way to reduce the mobility and bioavailability of the metallic pollutants in the soils, especially for the highly mobile Cd. However, for a correct assessment is necessary take into account the different nature of the contaminants (cation vs. anion), soil properties and experimental conditions.

Concerning the application of humic acids to industrial soil and sludge polluted by Pb and Zn, a general positive influence in term of Giant reed (*Arundo donax* L.) biomass, growth conditions and plant adaptability was observed. By contrast, the presence of humic acids did not appear to significantly affect the Pb and Zn mobility and bioavailability in both substrates. Other factors, mainly pH, could concur to this result. *A. donax,* despite the low metals bioavailability in the studied soil and sludge, resulted to be able in the phytoextraction of metals in all conditions, hence, useful for phytoremediation. The cultivation of plants with high biomass, like the studied plant, is feasible also in dismissed industrial lands, making this type of fields useful for bioenergy production.

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